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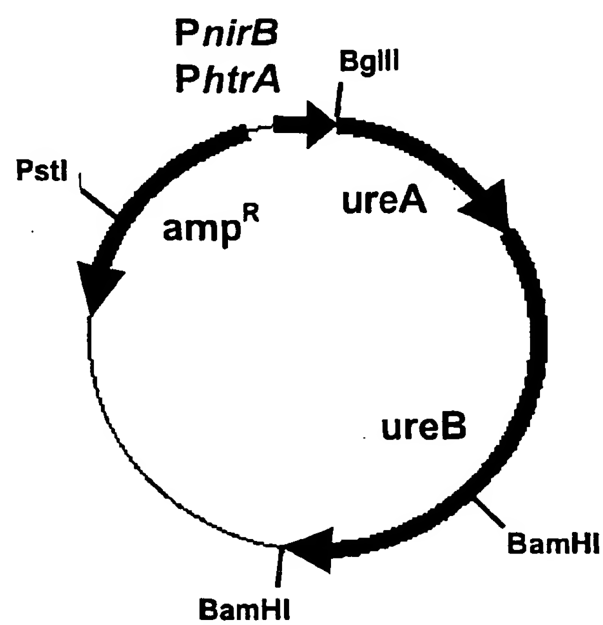
(54) **Title:** USE OF SALMONELLA VECTORS FOR VACCINATION AGAINST HELLICOBACTER INFECTION



(57) **Abstract:** The invention provides a method of immunization against Helicobacter, involving mucosal administration of an attenuated Salmonella vector including a nucleic acid molecule encoding a Helicobacter antigen, and parenteral administration of a soluble Helicobacter antigen, co-administered with a suitable parenteral adjuvant. Also provided by the invention are attenuated Salmonella vectors for use in this method.

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DNA adenine methylase **mutants of** Salmonella **typhimurium show defects in protein secretion, cell invasion, and M cell cytotoxicity.**

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Mutants of **Salmonella** typhimurium lacking DNA adenine **methylase** are attenuated for virulence in BALB/c mice. LD(50) values of a DNA adenine methylation (Dam)(-) mutant are at least 10(3)- to 10(4)-fold higher than those of the parental strain when administrated by oral or intraperitoneal routes. Dam(-) mutants are unable to proliferate in target organs but persist in low numbers in these locations. Efficient protection to challenge with the virulent parental strain is observed in mice infected with a Dam(-) mutant. Use of the ileal loop assay shows that Dam(-) mutants are less cytotoxic to M cells and fail to invade enterocytes. In the tissue culture model, lack of DNA adenine methylation causes reduced ability to invade nonphagocytic cells. In contrast, no effect is observed either in intracellular proliferation within nonphagocytic cells or in survival within macrophages. The invasion defect of Dam(-) mutants is correlated with a distinct pattern of secreted proteins, which is observed in both **PhoP** (+) and **PhoP** (-) backgrounds. Altogether, our observations suggest a multifactorial role of Dam methylation in **Salmonella** virulence.

Tags: Female; Research Support, Non-U.S. Gov't

Descriptors: *Bacterial Proteins--secretion--SE; *Intestinal Mucosa --microbiology--MI; * **Salmonella** typhimurium--enzymology--EN; *Site-Specific DNA-Methyltransferase (Adenine-Specific)--physiology--PH; Animals; DNA Methylation; Hela Cells; Humans; Mice; Mice, Inbred BALB C; Mutation; **Salmonella** typhimurium--metabolism--ME; **Salmonella** typhimurium--pathogenicity--PY; Site-Specific DNA-Methyltransferase (Adenine-Specific)--genetics--GE; Virulence

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An essential role for DNA adenine methylation in bacterial virulence.

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Salmonella typhimurium lacking DNA adenine **methylase** (Dam) were fully proficient in colonization of mucosal sites but showed severe defects in colonization of deeper tissue sites. These Dam- mutants were totally avirulent and were effective as live vaccines against murine typhoid fever. Dam regulated the expression of at least 20 genes known to be induced during infection; a subset of these genes are among those activated by the **PhoP** global virulence regulator. **PhoP** , in turn, affected Dam methylation at specific genomic sites, as evidenced by alterations in DNA methylation patterns. Dam inhibitors are likely to have broad antimicrobial action, and Dam- derivatives of these pathogens may serve as live attenuated vaccines.

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preferred embodiments the bacterial cell includes a second mutation which attenuates virulence.

In yet other preferred embodiments of the vaccine the two-component regulatory system is the phoP regulatory region, and the gene under the control of the two-component system is a phoP regulatory region regulated gene, e.g., a *prg* or *pag* gene, e.g., *pag*C. In preferred embodiments constitutive expression is the result of a change or mutation (preferably a non-revertible mutation) at the promoter of the regulated gene or of the phoP regulatory region, e.g., a mutation in the *pho*Q or the *pho*P gene, e.g., the *pho*P^{c} mutation.

In preferred embodiments of the vaccine the *Salmonella* cell includes a first mutation which attenuates virulence, e.g., a mutation in a *pho*P regulatory region gene, e.g., a mutation in the *pho*P or *pho*Q gene, e.g., *pho*P^{c}, or a mutation in a phoP regulatory region regulated gene, and a second mutation which attenuates virulence, e.g., a mutation in an *aro*matic amino acid synthetic gene, e.g., an *aro* gene, a mutation in a phoP regulatory region regulated gene, e.g., a mutation in a *prg* or *pag* locus, e.g., a *pag*C mutation.

In yet other preferred embodiments the bacterial cell includes a first mutation in a phoP regulatory region gene and a second mutation in an *aro*matic amino acid synthetic gene, e.g, an *aro* gene.

In another aspect, the invention features a vaccine, preferably a live vaccine, including a bacterial cell, the virulence of which is attenuated by a mutation in a gene under the control of a two-component regulatory system. In preferred embodiments the bacterial cell includes a virulence attenuating mutation in a second gene, e.g., in an *aro*matic amino acid synthetic gene, e.g., an *aro* gene.

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(54) Title: ANTI-*HELICOBACTER* VACCINE COMPOSITION FOR USE BY THE SUBDIAPHRAGMATIC SYSTEMIC ROUTE, AND COMBINED MUCOSAL/PARENTERAL IMMUNIZATION METHOD

(57) Abstract

The subject of the invention is the use of an immunogenic agent derived from *Helicobacter*, in the manufacture of a pharmaceutical composition intended for the induction of a T helper 1 (Th1) type immune response against *Helicobacter*, to prevent or treat a *Helicobacter* infection in a mammal. This is in particular achieved when the pharmaceutical composition is intended to be administered by the systemic or parenteral route, for example, in the part of the mammal situated under its diaphragm. Also included in the invention is a mucosal/parenteral immunization method for the prevention or treatment of *Helicobacter* infection.

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ANTI-*HELICOBACTER* VACCINE COMPOSITION FOR USE BY THE SUBDIAPHRAGMATIC SYSTEMIC ROUTE, AND COMBINED MUCOSAL/PARENTERAL IMMUNIZATION METHOD

The subject of the present invention is the specific use of a vaccine preparation intended to induce, in a mammal, a protective immune response against a pathogenic organism infecting the mucous membranes, in particular against *Helicobacter* bacteria.

Helicobacter is a bacterial genus characterized by Gram-negative helical bacteria. Several species colonize the gastrointestinal tract of mammals. There may be mentioned in particular *H. pylori, H. heilmanii, H. felis*, and *H. mustelae*. Although *H. pylori* is the species most commonly associated with human infections, in some rare cases, it has been possible to isolate in man *H. heilmanii* and *H. felis*. A bacterium of the *Helicobacter* type, *Gastrospirillum hominis*, has also been described in man.

Helicobacter infects more than 50% of the adult population in developed countries and nearly 100% of that of developing countries, thereby making it one of the predominant infectious agents worldwide.

H. pylori is so far exclusively found at the surface of the mucous membrane of the stomach in man and more particularly around the crater lesions of gastric and duodenal ulcers. This bacterium is currently recognized as the aetiological agent of antral gastritis and appears as one of the cofactors required for the development of ulcers. Moreover, it seems that the development of gastric carcinomas may be associated with the presence of *H. pylori*.

It therefore appears to be highly desirable to develop a vaccine intended to prevent or treat *Helicobacter* infections.

To date, several *Helicobacter* proteins have already been proposed as vaccinal antigens and the method of vaccination that is commonly recommended consists of

delivering the antigen at the level of the gastric mucous membrane, that is to say at the very site where the immune response is desired. To do this, oral administration was therefore selected.

Still with the same aim, induction of an immune response at the level of the stomach, it has been more recently proposed to deliver the antigen at a mucosal site other than the gastric mucous membrane, such as the nasal or rectal mucous membrane, for example (WO 96/31235). Lymphocytes stimulated by the antigen in a so-called inducer mucosal territory can migrate and circulate selectively so as to go and induce an immune response in other so-called effector mucosal territories.

A variant of these methods involves carrying out a first immunization by the systemic route before administering the antigen by the nasal route.

For administration by the mucosal route, the antigen, most often a bacterial lysate or a purified protein, is combined with an appropriate adjuvant such as cholera toxin (CT) or the heat-labile toxin (LT) from *E. coli*.

When administration by the mucosal route is used, the humoral response that is observed is predominantly of the IgA type. This indeed indicates that there has been a local immune response.

Some authors thought very early on that there was a good correlation between a strong response of the IgA type and a protective effect (Czinn *et al.*, Vaccine (1993) 11: 637). Others gave a more reserved opinion (Bogstedt *et al.*, Clin. Exp. Immunol. (1996) 105: 202). Although there is up until now no real certainty on this subject, the induction of antibodies that are in particular of the IgA type appears nonetheless desirable for most authors.

In general, the appearance of IgAs is indicative of the coming into play of a response on the part of the type 2 T helper lymphocytes (Th2 response).

Indeed, the stimulation of the T helper lymphocytes by a particular antigen makes it possible to obtain various subpopulations of T helper cells, characterized by different cytokine synthesis profiles.

The Th1 cells in particular produce selectively interleukin-2 (IL-2) and interferon-γ (IFN-γ), whereas the Th2 cells secrete preferably IL-4, IL-5, and IL-10. Because of their differentiated production of cytokines, these two types of T helper cells have distinct roles: the Th1 cells promote cell-mediated immunity, *i.a.*, an inflammatory-type response, whereas the Th2 cells stimulate humoral response of the IgA, IgE, and certain IgG subclass types. It is also known that the cytokines produced by mouse Th1 cells can stimulate antibody response and in particular that IFN-γ induces an IgG2a response. Thus, from the various studies in the prior art, the view emerges according to which the induction of a Th2 response characterized by the appearance of IgA is essential, if not enough, to obtain a protective effect.

Surprisingly, it has now been discovered that even if a Th2 response is not damaging, it is also necessary to induce a high Th1 response. Indeed, experimental results now demonstrate that a protective effect may be more easily correlated with a Th1 response than with a Th2 response.

Contrary to what was initially sought (D'Elios *et al.*, J. Immunol. (1997) 158: 962), the present application therefore reveals the importance of inducing an inflammatory-type Th1 response at the time of immunization, without which a protective effect cannot be observed.

Consequently, the subject of the present invention is:

(i) The use of an immunogenic agent derived from a microorganism capable of infecting the gastroduodenal mucous membrane of a mammal, *e.g.*, derived from *Helicobacter*, in the manufacture of a pharmaceutical composition intended for the induction of a Th1-type immune response against the said microorganism, *e.g.*, *Helicobacter*, for treating or preventing an infection, *e.g.*, a *Helicobacter* infection in a mammal; and

(ii) a method for preventing or treating an infection promoted by a microorganism capable of infecting the gastroduodenal mucous membrane of a mammal, *e.g.*, a *Helicobacter* infection, according to which there is administered to

the mammal, in one or more applications, at least one immunogenic agent derived from the said microorganism, *e.g.*, from *Helicobacter*, and by which a Th1-type immune response is induced against, *e.g.*, *Helicobacter*.

The induction of a useful Th1 response can be demonstrated for the purposes of the present invention by estimating the relative level of the Th1 response relative to the Th2 response by comparing, for example, the IgG2a and IgG1 levels induced in mice against *Helicobacter*, which are respectively indicative of the coming into play of the Th1 and Th2 responses. Indeed, the Th1 response which is sought is generally accompanied by a Th2 response. However, it is considered that the Th2 response should not be significantly predominant relative to the Th1 response. The IgG2a and IgG1 levels induced in mice can be assessed conventionally using an ELISA test, provided that the tests used for each of the two subisotypes are of the same sensitivity and, in particular, that the anti-IgG2a and anti-IgG1 antibodies are of the same affinity.

The quantities of IgG2a and IgG1 can be measured in particular using an ELISA test that is identical or similar to that described below. The wells of a polycarbonate ELISA plate are coated with 100 μl of a bacterial extract from *Helicobacter, e.g.*, *H. pylori*, at about 10 μg/ml in carbonate buffer. The ELISA plate is incubated for 2 hours at 37°C and then overnight at 4°C. The plate is washed with PBS buffer (phosphate buffered saline) containing 0.05% Tween 20 (PBS/Tween buffer). The wells are saturated with 250 μl of PBS containing 1% bovine serum albumin to prevent nonspecific binding of the antibodies. After incubating for one hour at 37°C, the plate is washed with PBS/Tween buffer. The antiserum collected from mice, a number of days after the latter have received the composition intended to induce a Th1-type immune response against *Helicobacter*, is serially diluted in PBS/Tween buffer. 100 μl of the dilutions are added to the wells. The plate is incubated for 90 minutes at 37°C, washed, and evaluated according to standard procedures. For example, a goat antibody to mouse IgG2a or IgG1, coupled to an

enzyme such as peroxidase, is used. The incubation in the presence of this antibody is continued for 90 minutes at 37°C. The plate is washed and then the reaction is developed with the appropriate substrate, for example, O-phenyldiamine dihydrochloride when the enzyme used is peroxidase. The reaction is evaluated by colorimetry by measuring the absorbance by spectrophotometry. The IgG2a or IgG1 titre of the antiserum corresponds to the reciprocal of the dilution giving an absorbance of 1.5 at 490 nm.

The induction of a useful Th1 response for the purposes of the present invention is marked by a ratio of the ELISA IgG2a:IgG1 titers in mice which should be greater than 1/100, 1/50, or 1/20, advantageously greater than 1/10, preferably greater than 1/3, most preferably greater than ½, 5, or 10. When this ratio is around 1, the Th1/Th2 response is said to be mixed or balanced. When the ratio is greater than or equal to 5, the Th1 response is then said to be preponderant.

The production of a Th1 (or Th2) response in mice is predictive of a Th1 (or Th2) response in man. Although it is easier to evaluate the type of response in mice, it can also be done in man by measuring the levels of cytokines specific for the Th1 response on the one hand and, on the other hand, for the Th2 response, which are subsequently induced. The Th1 and Th2 responses can be evaluated directly in man relative to each other on the basis of the levels of cytokines specific for the two types of response (see above), *e.g.*, on the basis of the IFN-γ/IL-4 ratio.

Alternatively, if the assay method described above is used, it is possible to predict that the ELISA titre that reflects the quantity of IgG2a should be equal to or greater than 10,000, preferably equal to or greater than 100,000, in a particularly preferred manner, equal to or greater than 1,000,000; this then means that the Th1 response is significant.

The mammal for which the pharmaceutical composition or the method is intended is advantageously a primate, preferably a human.

It is possible to induce a Th1 response against *Helicobacter* by adjusting a

number of factors, such as, for example, the route of administration. It has indeed been demonstrated that by using the systemic or parenteral route, a level of protection can be obtained that is similar to or greater than that observed when the mucosal route is used.

Accordingly, the subject of the invention is in particular:

(i) the use of an immunogenic agent derived from *Helicobacter*, in the manufacture of a pharmaceutical composition intended to be administered by the systemic or parenteral route in the part of a mammal, especially a primate, situated under its diaphragm, for treating or preventing a *Helicobacter* infection; and

(ii) a method for preventing or treating a *Helicobacter* infection in a mammal, according to which there is administered to the said mammal, in one or more applications, by the systemic or parenteral route, at least one immunogenic agent derived from *Helicobacter*.

As regards the method, it is indicated that, advantageously, the administration of the immunogenic agent by the systemic or parenteral route is repeated once or several times, preferably at least twice, for the desired immune response to be induced. A preferred method by which a protective effect is obtained is in particular a method according to which the immunogenic agent is administered exclusively by the systemic or parenteral route (strict systemic route). "A method in which the administration of the immunogenic agent is carried out by the strict systemic route" is defined as a method not using a route of administration other than the systemic route. For example, a method in which the immunogenic agent is administered by the systemic route and by the mucosal route does not correspond to the definition given above. In other words, "a method in which the administration of the immunogenic agent is carried out by the strict systemic route" should be understood to mean a method in which the immunogenic agent is administered by the systemic route excluding any other route, in particular the mucosal route.

Still as regards the method, the administration by the systemic or parenteral

route is advantageously carried out in the subdiaphragmatic part of the mammal.

The immunogenic agent derived from *Helicobacter* is advantageously selected from a preparation of inactivated *Helicobacter* bacteria, a *Helicobacter* cell lysate, a peptide and a polypeptide from *Helicobacter* in purified form. The immunogenic agent can also be a polynucleotide molecule, especially a DNA molecule including a sequence encoding a peptide or a polypeptide from *Helicobacter* placed under the control of elements necessary for its expression in a mammalian cell, or alternatively a viral vaccinal vector including a sequence encoding a peptide or a polypeptide from *Helicobacter* placed under the control of elements necessary for its expression in a mammalian cell.

For the purposes of the present invention, a preparation of inactivated bacteria can be obtained according to conventional methods well known to persons skilled in the art. Likewise for a bacterial lysate. A dose of inactivated bacteria or cell lysate, appropriate for prophylactic or therapeutic purposes, can be determined by persons skilled in the art and depends on a number of factors, such as the individual for whom the vaccine is intended, *e.g.*, the individual's age, the antigen itself, the route and mode of administration, the presence/absence or the type of adjuvant, as can be determined by persons skilled in the art. In general, it is indicated that an appropriate dose is from about 50 μg to 1 mg at about 1 mg of lysate.

A peptide or a polypeptide derived from *Helicobacter* can be purified from *Helicobacter* or obtained by genetic engineering techniques or alternatively by chemical synthesis. The latter process is advantageous in the case of peptides. "Peptide" is any amino acid chain of less than about 50 amino acids. When the size is greater, the term "polypeptide," which is also interchangeable with the term "protein," is used. A useful peptide or polypeptide for the purposes of the present invention can be identical or similar to that which exists under natural conditions. It is similar in that it is capable of inducing an immune response of the same type but it can include certain structural variations such as, for example, a mutation, the addition of a residue

of a lipid nature, or, alternatively, it can be in fusion polypeptide or peptide form.

An appropriate dose of peptide or polypeptide for prophylactic or therapeutic purposes can be determined by persons skilled in the art and depends on a number of factors, such as the individual for whom the vaccine is intended, *e.g.*, the age of the individual, the antigen itself, the route and mode of administration, the presence/absence or the type of adjuvant, as can be determined by persons skilled in the art. In general, it is indicated that an appropriate dose is from about 10 μg to about 1 mg, preferably at about 100 μg.

The DNA molecule can advantageously be a plasmid that is incapable both of replicating and of substantially integrating into the genome of a mammal. The above-mentioned coding sequence is placed under the control of a promoter allowing expression in a mammalian cell. This promoter can be ubiquitous or specific for a tissue. Among the ubiquitous promoters, there may be mentioned the Cytomegalovirus early promoter (described in U.S. Patent No. 4,168,062) and the Rous sarcoma virus promoter (described in Norton & Coffin, Molec. Cell. Biol. (1985) 5: 281). The desmin promoter (Li *et al.*, Gene (1989) 78: 244443; Li & Paulin, J. Biol. Chem. (1993) 268: 10403), which is a selective promoter, allows expression in muscle cells and also in skin cells. A promoter specific for muscle cells is, for example, the promoter of the myosin or dystrophin gene. Plasmid vectors that can be used for the purposes of the present invention are described, *i.a.*, in WO 94/21797 and Hartikka *et al.*, Human Gene Therapy (1996) 7: 1205.

In a useful pharmaceutical composition for the purposes of the present invention, the nucleotide molecule, *e.g.*, the DNA molecule, can be formulated or otherwise. The choice of formulation is highly varied. The DNA can be simply diluted in a physiologically acceptable solution with or without carrier. When the latter is present, it can be isotonic or weakly hypertonic and can have a low ionic strength. For example, these conditions can be fulfilled by a sucrose solution, *e.g.*, at 20%.

Alternatively, the polynucleotide can be combined with agents that promote entry into the cell. This can be (i) a chemical agent that modifies cell permeability, such as bupivacaine (see, for example, WO 94/16737), or (ii) an agent that is combined with the polynucleotide and that acts as a vehicle facilitating the transport of the polynucleotide. The latter may be in particular cationic polymers, *e.g.*, polylysine or a polyamine, *e.g.*, derivatives of spermine such as spermidine (see WO 93/18759). This can also be fusogenic peptides, *e.g.*, GALA or Gramicidin S (see WO 93/19768) or, alternatively, peptides derived from viral fusion proteins.

This can also be anionic or cationic lipids. The anionic or neutral lipids have been known for a long time to be capable of serving as transporting agents, for example, in the form of liposomes, for a large number of compounds, including polynucleotides. A detailed description of these liposomes, of their constituents, and of the processes for their manufacture is, for example, provided by *Liposomes: A Practical Approach*, RPC New Ed., IRL press (1990).

The cationic lipids are also known and are commonly used as transporting agents for polynucleotides. There may be mentioned for example Lipofectin™ also known by the name DOTMA (N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride), DOTAP (1,2-bis(oleyloxy)-3-(trimethyl-ammonio)propane), DDAB (dimethyldioctadecylammonium bromide), DOGS (dioctadecylamidoglycyl spermine), and cholesterol derivatives, such as DC-chol (3-beta-(N-(N',N'-dimethylaminoethane) carbamoyl) cholesterol). A description of these lipids is provided by EP 187,702, WO 90/11092, U.S. Patent No. 5,283,185, WO 91/15501, WO 95/26356, and U.S. Patent No. 5,527,928. The cationic lipids are preferably used with a neutral lipid such as DOPE (dioleylphosphatidylethanolamine) as is, for example, described in WO 90/11092.

Gold or tungsten microparticles can also be used as transporting agents, as described in WO 91/359, WO 93/17706, and Tang *et al.*, Nature (1992) 356: 152. In this particular case, the polynucleotide is precipitated on the microparticles in the

presence of calcium chloride and spermidine, and then the whole is administered by a high-speed jet into the dermis or into the epidermis using an apparatus with no needle, such as those described in U.S. Patent Nos. 4,945,050 and 5,015,580, and WO 94/24243.

The quantity of DNA that can be used to vaccinate an individual depends on a number of factors such as, for example, the strength of the promoter used to express the antigen, the immunogenicity of the product expressed, the condition of the mammal for whom the administration is intended (*e.g.*, the weight, age, and general state of health), the mode of administration, and the type of formulation. It is indicated in particular that the administration by the intramuscular route requires a larger quantity of DNA than the administration by the intradermal route using an apparatus with no needle. In general, an appropriate dose for prophylactic or therapeutic use in an adult of the human species is from about 1 μg to about 5 mg, preferably from about 10 μg to about 1 mg, most preferably from about 25 μg to about 500 μg.

Vaccinal vectors are among the immunogenic agents mentioned above. Adenoviruses and poxviruses in particular are among the vectors of viral origin. An example of a vector derived from an adenovirus, as well as a method for constructing a vector capable of expressing a DNA molecule encoding a useful peptide or polypeptide for the purposes of the present invention, are described in U.S. Patent No. 4,920,209. Poxviruses that can be used likewise are, for example, the vaccinia and canarypox viruses. They are described respectively in U.S. Patents Nos. 4,722,848 and 5,364,773 (see also, *e.g.*, Tartaglia *et al.*, Virology (1992) 188: 217 and Taylor *et al.*, Vaccine (1995) 13: 539). Poxviruses capable of expressing a useful peptide or polypeptide for the purposes of the present invention can be obtained by homologous recombination, as described in Kieny *et al.*, Nature (1984) 312: 163, such that the DNA fragment encoding the peptide or polypeptide is placed under conditions appropriate for its expression in mammalian cells. A bacterial vector such as the bile

Calmette-Guérin bacillus can also be used.

In general, the dose of a viral vector intended for prophylactic or therapeutic purposes can be from about 1×10^4 to about 1×10^{11}, advantageously from about 1×10^7 to about 1×10^{10}, and preferably from about 1×10^7 to about 1×10^9 plaque forming units *per* kilogram.

The immunogenic agent derived from *Helicobacter* can be any polypeptide from *Helicobacter*, *e.g.*, *H. pylori*. This can be in particular a polypeptide present in the cytoplasm, a polypeptide of the inner or outer membrane, or a polypeptide secreted in the external medium. Numerous polypeptides from *Helicobacter* have already been described in the literature, either with reference to their amino acid sequence deduced from the sequence of the cloned or identified corresponding gene, or with reference to a purification process that makes it possible to obtain them in a form isolated from the rest of their natural environment. As a guide, the following documents are mentioned in particular: WO 94/26901 and WO 96/34624 (HspA), WO 94/09023 (CagA), WO 96/38475 (HpaA), WO 93/181150 (cytotoxine), WO 95/27506 and Hazell *et al.*, J. Gen. Microbiol. (1991) 137: 57 (catalase), FR 2 724 936 (membrane receptor for human lactoferrin), WO 96/41880 (AlpA), EP 752 473 (FibA) and O'Toole *et al.*, J. Bact. (1991) 173: 505 (TsaA). Other polypeptides are also described in WO 96/40893, WO 96/33274, WO 96/25430, and WO 96/33220. A useful polypeptide for the purposes of the present invention can be identical or similar to one of those cited as a reference insofar as it is capable of promoting an immune response against *Helicobacter*. In order to meet this last condition, the immunogenic agent can also be a peptide derived from a polypeptide cited as a reference.

Advantageously, a polypeptide selected from the UreA and UreB subunits of *Helicobacter* urease is used (see WO 90/4030). Preferably, both are used, combined in urease apoenzyme form or alternatively in multimeric form (see WO 96/33732).

Likewise, a useful vaccinal vector or DNA molecule for the purposes of the present invention includes a sequence that can encode any polypeptide or peptide

described above.

A DNA molecule, or preferably a viral vaccinal vector, can also include a sequence encoding a cytokine, for example, a lymphokine, such as interleukin-2 or interleukin-12, under the control of elements appropriate for expression in a mammalian cell. An alternative to this option also consists in adding to a useful pharmaceutical composition for the purposes of the present invention comprising a DNA molecule or a vector, another molecule, or viral vector encoding a cytokine.

A useful pharmaceutical composition for the purposes of the present invention can contain a single immunogenic agent or several. For example, an advantageous composition can comprise UreA and UreB, *e.g.*, in apoenzyme form, as well as one or more other polypeptides selected in particular from those mentioned above. Likewise, when a DNA molecule or a vaccinal vector is involved, the composition can contain several of them, each encoding a particular polypeptide or a single DNA molecule or vaccinal vector encoding several peptides or polypeptides.

A useful pharmaceutical composition for the purposes of the present invention can, in addition, contain compounds other than the immunogenic agent itself, the nature of these compounds depending, to a certain extent, on the nature of the immunogenic agent, inactivated bacteria, cell lysate, peptide, or polypeptide, DNA molecule, or vaccinal vector. Thus, as has already been seen above, when a DNA molecule is involved, the pharmaceutical composition can include various formulation agents. A composition can also include an appropriate adjuvant for administration by the systemic or parenteral route, *e.g.*, an aluminum compound, such as aluminum hydroxide, aluminum phosphate, or aluminum hydroxyphosphate. In general, it is indicated that inactivated bacteria may not require the addition of an adjuvant. The same is true as regards the DNA molecules. On the other hand, the presence of an adjuvant is preferable when the immunogenic agent is a bacterial lysate or a purified peptide or polypeptide. Finally, when the immunogenic agent is a vaccinal vector, the use thereof is preferably avoided so that the immune response towards the vector itself

remains minimal.

In addition to the aluminum compounds, a large number of appropriate adjuvants for administration by the systemic or parenteral route exist in the state of the art among which persons skilled in the art are capable of selecting the one that best corresponds to their needs; in particular a compound capable of promoting the induction of a Th1-type immune response or a balanced response of the Th1 + Th2 type. As a guide, there can be mentioned in particular liposomes; ISCOMS; microspheres; protein chochleates; vesicles consisting of nonionic surfactants; cationic amphiphilic dispersions in water; oil/water emulsions; muramidyldipeptide (MDP) and its derivatives such as glucosyl muramidyldipeptide (GMDP), threonyl-MDP, murametide and murapalmitin; and QuilA and its subfractions; as well as various other compounds such as monophosphoryl-lipid A (MPLA) major lipopolysaccharide from the wall of a bacterium, for example of *E. coli, Salmonella minnesota, Salmonella typhimurium*, or *Shigella flexneri*; algan-glucan; gamma-inulin; calcitriol; and loxoribine.

Useful liposomes for the purposes of the present invention can be selected in particular from pH-sensitive liposomes, such as those formed by mixing cholesterol hemisuccinate (CHEMS) and dioleyl phosphatidyl ethanolamine (DOPE); liposomes containing cationic lipids recognized for their fusiogenic properties, such as 3-beta-(N-(N',N'-dimethylamino-ethane)carbamoyl)cholesterol (DC-chol) and its equivalents, which are described in U.S. Patent No. 5,283,185 and WO 96/14831, dimethyldioctadecylammonium bromide (DDAB) and the BAY compounds described in EP 91645 and EP 206 037, for example Bay R1005 (N-(2-deoxy-2-L-leucylamino-beta-D-glucopyranosyl)-N-octa-decyldodecanoylamide acetate; and liposomes containing MTP-PE, a lipophilic derivative of MDP (muramidyldipeptide). These liposomes are useful for adding as adjuvant to all the immunogenic agents cited.

Useful ISCOMs for the purposes of the present invention can be selected in particular from those compounds of QuilA or of QS-21 combined with cholesterol and

optionally also with a phospholipid such as phosphatidylcholine. These are particularly advantageous for the formulation of the lipid-containing antigens.

Useful microspheres for the purposes of the present invention can be formed in particular from compounds such as polylactide-co-glycolide (PLAGA), alginate, chitosan, polyphosphazene, and numerous other polymers.

Useful protein chochleates for the purposes of the present invention can be selected in particular from those formed from cholesterol and optionally an additional phospholipid, such as phosphatidylcholine. These are especially advantageous for the formulation of the lipid-containing antigens.

Useful vesicles consisting of nonionic surfactants for the purposes of the present invention can be in particular formed by a mixture of 1-mono-palmitoyl glycerol, cholesterol, and dicetylphosphate. They are an alternative to the conventional liposomes and can be used for the formulation of all the immunogenic agents cited.

Useful oil/water emulsions for the purposes of the present invention can be selected in particular from MF59 (Biocine-Chiron), SAF1 (Syntex), and the montanides ISA51 and ISA720 (Seppic).

A useful adjuvant for the purposes of the present invention can also be a fraction derived from the bark of the South American tree *Quillaja Saponaria Molina*; for example, QS-21, a fraction purified by HPLC chromatography as is described in U.S. Patent No. 5,057,540. Since some toxicity may be associated with QS-21, it may be advantageous to use the latter in liposomes especially based on sterol, as is described in WO 96/33739.

Finally, an adjuvant effect can also be obtained by adding lipid to the useful peptide or polypeptide for the purposes of the present invention. The combination, by covalent bonding, of such a peptide or polypeptide with a lipid or a lipid-containing compound capable of promoting the induction of a Th1-type immune response, so as to form a lipid-containing lipopeptide or polypeptide conjugate, can be achieved in

various ways known to persons skilled in the art. For example, it is possible to use one of the compounds described in EP 431 327 such as N-palmitoyl-S-2,3-(bispalmitoyloxy) propylcysteinylseryl serine (Pam_3CSS), which is coupled by known processes to the N-terminal end of the peptide or polypeptide.

The therapeutic or prophylactic efficacy of a method or of a use according to the invention can be evaluated according to standard methods, *e.g.*, by measuring the induction of an immune response or the induction of a therapeutic or protective immunity using, *e.g.*, the mouse/*H. felis* model and the procedures described in Lee *et al.*, Eur. J. Gastroenterology & Hepatology (1995) 7: 303 or Lee *et al.*, J. Infect. Dis. (1995) 172: 161. Persons skilled in the art will realize that *H. felis* can be replaced in the mouse model by another *Helicobacter* species. For example, the efficacy of an immunogenic agent derived from *H. pylori* is preferably evaluated in a mouse model using an *H. pylori* strain adapted to mice. The efficacy can be determined by comparing the level of infection in the gastric tissue (by measuring the urease activity, the bacterial load, or the condition of the gastritis) with that in a control group. A therapeutic effect or a protective effect exists when the infection is reduced compared with the control group.

A useful pharmaceutical composition for the purposes of the present invention can be manufactured in a conventional manner. In particular, it can be formulated with a pharmaceutically acceptable carrier or diluent, *e.g.*, water or a saline solution. In general, the diluent or carrier can be selected according to the mode and route of administration and according to standard pharmaceutical practices. Appropriate carriers or diluents, as well as what is essential for the preparation of a pharmaceutical composition, are described in *Remington's Pharmaceutical Sciences*, a standard reference book in this field.

The methods according to the invention, as well as the compositions useful for these purposes, can be used to treat or prevent, *i.a.*, *Helicobacter* infections and consequently the gastroduodenal diseases associated with these infections, including

acute, chronic, or atrophic gastritis, and peptic ulcers, *e.g.*, gastric or duodenal ulcers.

The systemic route that is used can be the parenteral route, which can itself be chosen from the intravenous, intramuscular, intradermal, intraepidermal, and subcutaneous routes; the latter four being however preferred to the intravenous route. The intramuscular and subcutaneous routes are particularly recommended. In all cases, the use that will be made of the pharmaceutical composition can call into play a site of administration situated under the diaphragm of an individual. The dorsolumbar region constitutes, for example, an appropriate site of administration.

To obtain a protective or therapeutic effect, the operation that consists of administering, for example, by the subdiaphragmatic systemic route, a useful pharmaceutical composition for the purposes of the present invention can be repeated once or several times, leaving a certain time interval between each administration; which interval is of the order of a week or a month. Its precise determination is within the capability of persons skilled in the art and can vary according to various factors, such as the nature of the immunogenic agent, the age of the individual, and the like. In this particular case, the administration is said to be of the strict systemic type. By way of a nonlimiting illustration, there may be mentioned a vaccination scheme that consists of administering the urease apoenzyme three times by the subcutaneous route, in the dorsolumbar region, with an interval of two to four weeks between each administration.

According to an alternative mode, it is possible to envisage operating in a strict systemic mode of administration, but using immunogenic agents that vary during the administrations constituting the steps of the vaccination procedure. By way of a nonlimiting illustration, there may be mentioned a vaccination scheme by the strict systemic route, in three steps: a first administration (priming) consists of administering a pox vector encoding, *e.g.*, UreA and UreB, followed by two consecutive administrations (boosters) of the urease apoenzyme.

In general, the subject of the invention is therefore also a pharmaceutical

composition intended to treat or prevent a *Helicobacter* infection which includes, for consecutive administration, several products, each of the products being formulated so as to be administered by the subdiaphragmatic systemic route and containing an immunogenic agent derived from *Helicobacter* selected independently from a preparation of inactivated *Helicobacter* bacteria, a *Helicobacter* cell lysate, a peptide, a polypeptide from *Helicobacter* in purified form, a DNA molecule comprising a sequence encoding a peptide or a polypeptide from *Helicobacter* placed under the control of the elements necessary for its expression, and a vaccinal vector including a sequence encoding a peptide or a polypeptide from *Helicobacter* placed under the control of the elements necessary for its expression, preferably provided that when a first product contains a peptide or a polypeptide and a second product contains a DNA molecule or a vaccinal vector, the coding sequence of the DNA molecule or of the vaccinal vector encodes the peptide or polypeptide contained in the first product.

Finally, an alternative vaccination procedure comprising several administrations staggered over time, *e.g.*, within time intervals of the order of a week or a month, to be determined by persons skilled in the art, can include a first administration by the subdiaphragmatic systemic route and a second administration by the mucosal route other than the intranasal route, *e.g.*, by the ocular, oral, *e.g.*, buccal or gastric, pulmonary, intestinal, rectal, vaginal, or urinary route. By way of a nonlimiting illustration, there can be mentioned a vaccination procedure that consists of administering a DNA molecule or a vaccinal vector by the subdiaphragmatic systemic route and then in administering a polypeptide by the gastric route, the DNA molecule or the vaccinal vector preferably encoding the polypeptide administered by the gastric route.

In general, the subject of the invention is therefore also a pharmaceutical composition intended to treat or prevent a *Helicobacter* infection that contains, for consecutive administration, several products; one of the products being formulated so as to be administered by the subdiaphragmatic systemic route and another product

being formulated so as to be administered by a mucosal route other than the intranasal route; each of the products containing an immunogenic agent derived from *Helicobacter* selected independently from a preparation of inactivated *Helicobacter* bacteria, a *Helicobacter* cell lysate, a peptide, a polypeptide from *Helicobacter* in purified form, a DNA molecule including a sequence encoding a peptide, or a polypeptide from *Helicobacter* placed under the control of the elements necessary for its expression and a vaccinal vector including a sequence encoding a peptide or a polypeptide from *Helicobacter* placed under the control of the elements necessary for its expression, preferably provided that when a first product contains a peptide or a polypeptide and a second product contains a DNA molecule or a vaccinal vector, the coding sequence of the DNA molecule or of the vaccinal vector encodes the peptide or polypeptide contained in the first product.

A vaccinal vector contained in a product intended to be administered by the mucosal route can be chosen from those described above. In addition, it can be selected from bacterial vectors such as *Shigella, Salmonella, Vibrio cholerae, Lactobacillus*, and *Streptococcus*.

Nontoxic mutant strains of *Vibrio cholerae* that can be useful as live vaccine vectors are described, for example, in Mekalanos *et al.*, Nature (1983) 306: 551 and U.S. Patent No. 4,882,278 (strain in which a substantial part of the region encoding each of the two alleles *ctxA* has been deleted so that no functional toxin can be produced); WO 92/11354 (strain in which the *irgA* locus is inactivated by mutation; this mutation may be combined in the same strain with *ctxA* mutations); and WO 94/1533 (mutant obtained by deletion lacking functional *ctxA* and *attRS1* sequences). These strains can be modified genetically to express heterologous antigens as described in WO 94/19482.

Attenuated strains of *Salmonella typhimurium*, genetically modified or otherwise for the recombinant expression of heterologous antigens, as well as their use as vaccines, are described in Nakayama *et al.*, BioTechnology (1988) 6: 693 and WO

are
aroA phoP pagC cya crp

92/11361.

Other bacteria useful as vaccinal vectors are described in High *et al.*, EMBO (1992) 11: 1991 and Sizemore *et al.*, Science (1995) 270: 299 (*Shigella flexneri*); Medaglini *et al.*, Proc. Natl. Acad. Sci. USA (1995) 92: 6868 (*Streptococcus gordonii*); and Flynn J.L., Cell. Mol. Biol. (1994) 40 (suppl. I): 31, WO 88/6626, WO 90/0594, WO 91/13157, WO 92/1796, and WO 92/21376 (Calmette-Guérin bacillus).

In bacterial vectors, the DNA sequence encoding a peptide or polypeptide from *Helicobacter* can be inserted into the bacterial genome or alternatively remain in the free state, carried by a plasmid. Obviously, this sequence is placed under the control of the elements necessary for its expression in the bacterial vector.

These bacterial vectors for administration by the mucosal route can be used in combination with an appropriate adjuvant. Such adjuvants may be chosen from bacterial toxins, *e.g.*, the cholera toxin (CT), the *E. coli* heat-labile toxin (LT), the *Clostridium difficile* toxin, and the *Pertussis* toxin (PT), or combinations, subunits, toxoids, or mutants that are derived therefrom. For example, it is possible to use a purified preparation of the native cholera toxin B subunit (CTB). Fragments, homologues, derivatives, and fusions of these toxins are equally suitable provided they retain the adjuvant activity. Preferably, a mutant is used whose toxicity is reduced. Such mutants are described in, *e.g.*, WO 95/17211 (mutant CT Arg-7-Lys), WO 96/6627 (mutant LT Arg-192-Gly), and WO 95/34323 (mutant PT Arg-9-Lys and Glu-129-Gly). Other LT mutants that can also be used carry at least one of the following mutations: Ser-63-Lys, Ala-69-Gly, Glu-110-Asp, and Glu-112-Asp.

Other compounds, such as MPLA, PLGA, DC-chol, and QS-21 can also be used as adjuvants for the mucosal route.

The invention also includes immunization methods for treating or preventing *Helicobacter* (*e.g.*, *H. pylori*) infection that involve mucosal (*e.g.*, oral, intranasal, intragastric, pulmonary, intestinal, rectal, ocular, vaginal, or urinary tract) administration, followed by parenteral (*e.g.*, intramuscular, subcutaneous, intradermal,

intramuscular, intravenous, or intraperitoneal). In one example of these methods, mucosal administration is carried out to prime an immune response to an antigen, and parenteral administration is carried out to boost the immune response to the antigen. Other examples of these methods involve alternating parenteral and mucosal administrations, for example, the following pattern can be used: intramuscular administration, combined intragastric + intranasal administration, intramuscular administration, and combined intragastric + intranasal administration. Antigens, formulations, adjuvants, administration regimens, specific mucosal and parenteral routes, and dosages to be used can readily be determined by one skilled in the art. Specific examples of these parameters that can be adapted for use in these methods are provided above.

In the description above, reference was made essentially to *Helicobacter* infections and to the means for combating them by way of prevention and prophylaxis. However, it should be understood that the principles and methods stated above can be applied *mutatis mutandis* to any other infection induced by any microorganism whose seat is the stomach, the duodenum or the intestine.

It is specified, in addition, that all the documents published and cited in the present application are incorporated by reference.

The invention is illustrated below with reference to the following figures.

Figure 1 refers to Example 1 and presents a study of the local response in the salivary glands (Figure 1A) and in the stomach (Figure 1B) evaluated by ELISPOT by measuring the quantity of anti-urease IgA induced, expressed as spots/10^6 cells (Figure 1A) or as number of responding mice, exhibiting more than 2 IgA spots/mouse, (Figure 1B), after (a) administration of urease at D0 by the subcutaneous route (SC) in the left posterior sublumbar part [(a) and (c)] or in the neck [(b) and (d)], followed by a booster by the nasal route (N) and intragastric route (IG), at D28 [(a) and (b)] or at D28 and D56 [(c) and (d)].

Figure 2 refers to Example 1 and presents the levels of urease activity after a

challenge, measured 4 hours after sacrificing mice which have received 3 times, on D0, D28 and D56, an inactivated bacterial preparation by the intragastric route [(a) and (c)] or subcutaneous route in the left posterior sublumbar part (b). In experiment (c), 10 μg of cholera toxin were added to the bacterial preparation. Experiments (d) and (e) correspond respectively to the positive and negative controls.

Figure 3 refers to Example 1 and presents the levels of urease activity after a challenge, measured 4 hours after sacrificing mice which have received 3 times, on D0, D28, and D56: (a) a urease preparation encapsulated at about 80% in DC-chol liposomes, in the dorsolumbar muscles; or (b) a urease preparation with cholera toxin adjuvant, by the intragastric route. Experiments (c) and (d) correspond respectively to the positive and negative controls.

Figure 4 refers to Example 1 and presents the levels of urease activity after a challenge measured 4 hours after sacrificing mice which have received 3 times, on D0, D28, and D56: (a) a urease preparation with cholera toxin adjuvant, by the intragastric route or (b) a urease preparation with QS-21 adjuvant, by the subcutaneous route in the left posterior sublumbar part. Experiments (c) and (d) correspond respectively to the positive and negative controls.

Figure 5 presents the quantities of serum immunoglobulins induced in monkeys subjected to the immunization procedures described in Example 2, and expressed as ELISA titre. A control group comprising 4 monkeys and three test groups are formed, each of the test groups comprising 8 monkeys; each test group is divided into two subgroups of 4 monkeys, one receiving only the inactivated *H. pylori* preparation (1, 2, and 3) and the other receiving the inactivated *H. pylori* preparation and recombinant urease (1u, 2u, and 3u). Group 1 and 1u corresponds to the administration procedure [nasal + intragastric, 4 times]; group 2 and 2u corresponds to the administration procedure [intramuscular, 4 times]; group 1 and 1u corresponds to the administration procedure [nasal + intragastric, intramuscular, nasal + intragastric, intramuscular]. The ELISA titre is measured three times: a first time at D0 (white band), a second

time at D42 (shaded band), a third time at D78 (black band).

Figures 6A and 6B show the urease activity (Figure 6A) measured after 4 hours (OD_{550} nm) using the Jatrox test (Procter & Gamble) and the bacterial load in mice infected with *H. pylori* and then submitted to various treatments A - H [A: LT + urease, orally; B: QS-21 + urease, parenterally in the neck; C: QS-21 + urease, parenterally in the lumbar region; D: QS-21 alone, sub-cutaneously in the lumbar region; E: Bay R1005 + urease, parenterally in the neck; F: Bay R1005 + urease, parenterally in the lumbar region; G: Bay R1005 alone, sub-cutaneously in the lumbar region (control); H: saline, sub-cutaneously in the lumbar region (positive control)]. I represents the negative control.

Figure 7 presents the results of immunization of mice with a mucosal prime/parenteral boost strategy with urease induced the most efficacious protection against challenge with *H. pylori*. Mice were immunized either orally with 25 μg urease + 5 μg LT or parenterally with 10 μg urease with or without 100 μg alum adjuvant. The mice were primed with orally administered urease + LT, 2 booster doses were administered three weeks apart by either the parenteral or oral route, as shown in the figure. Mice were challenged with *H. pylori* two weeks after the last immunization and euthanized 2 weeks after challenge. At necropsy, one-third of the stomach, dissected longitudinally, was homogenized and cultured for *H. pylori*.

Figure 8 shows the effect of urease immunization on experimental challenge of rhesus monkeys with *H. pylori*. Monkeys were immunized with urease by parenteral routes (100 μg urease + 1 mg alum or 800 μg Bay) or by a mucosal prime (orally administered 4 mg urease +100 μg LT)/parenteral boost (urease + alum) strategy with 3 doses administered every 3 weeks followed by a fourth dose administered 20 weeks after the first priming dose. Monkeys were challenged one week after the last booster dose. The monkeys were euthanized 10 weeks after challenge, 10 punch biopsies per animal were harvested from the stomach and cultured to determine *H. pylori* colonization. Each symbol above represents the mean CFU of 10 sites cultured per

monkey. The line represents the median CFU for the treatment group.

Figure 9 presents gastritis scores in immunized and unimmunized rhesus monkeys following challenge with *H. pylori*. Monkeys were orally immunized with a priming dose of 4 mg urease + 100 μg LT followed 3 weeks later with 2 parenteral administered 20 weeks after the first priming dose. Monkeys were challenged one week after the last booster dose. The monkeys were euthanized 10 weeks after challenge, 2 cm^2 sections were taken from the corpus, antrum and corporal-antral junction, fixed in 10% buffered formalin, embedded in paraffin and sections stained with H & E. Gastritis, typified by infiltration of lymphocytes, plasma cells, and polymorphonuclear cells, was scored by microscopic examination of stained sections. Each symbol above represents the mean gastritis score of the 3 regions from each monkey.

Figure 10 presents epithelial changes in immunized and unimmunized rhesus monkeys following challenge with *H. pylori*. Monkeys were orally immunized with a priming dose of 4 mg urease + 100 μg LT followed 3 weeks later with 2 parenteral doses of 100 μg urease + 1 mg alum every 3 weeks and 1 parenteral dose of urease + alum administered 20 weeks after the first priming dose. Monkeys were challenged one week after the last booster dose. The monkeys were euthanized 10 weeks after challenge, 2 cm^2 sections were taken from the corpus, antrum and corporal-antral junction, fixed in 10% buffered formalin, embedded in paraffin and sections stained with H & E. Epithelial changes, defined as metaplasia, atrophy and/or hyperplasia, was scored by microscopic examination of stained sections. Each symbol above represents the mean gastritis score of the 3 regions from each monkey.

Example 1: Immunization studies in mice

1A - Materials and methods

Mice

6/8-week old female Swiss mice were provided by Janvier (France). During

the whole experiment, sterilized materials were used; the cages were protected by "isocaps;" the mice were fed with filtered water and irradiated food.

Administration procedure

During each experiment, the mice received 3 doses of the same product; each dose at 28-day intervals (days 0, 28, and 56). The administration of the product was carried out by the nasal route (up to 50 μl on waking mice), by the oral route (300 μl in 0.2 M $NaHCO_3$ by gastric gavage), or by the subcutaneous route (300 μl under the skin of the neck or under the skin on the left side of the lumbar region). In some cases, an intramuscular inoculation was carried out (50 μl) in the dorsolumbar muscles of anaesthetized mice. Ten μg of urease were administered by the nasal, subcutaneous or intramuscular route, and 40 μg by the oral route. As regards the inactivated bacterial preparation, 400 μg of cells were administered by the subcutaneous route or by the oral route.

Antigens and adjuvants

The *H. pylori* urease apoenzyme was expressed in *E. coli* and purified as has been described in Example 5 of WO 96/31235. In the remainder of the text, the simple term of urease is used to designate this apoenzyme.

A preparation of inactivated *H. pylori* bacteria (WC) was prepared as follows: a bottle of frozen bacteria ATCC 43579 is diluted in a two-phase medium in a 75 cm^2 flask (Costar). This medium is composed of a solid constituent (10 ml Columbia agar (BioMérieux) + 6% sheep blood (BioMérieux)) and a liquid constituent (3 ml of TSB, BioMérieux). The flask is placed in a generbag containing a microaer (BioMérieux) and incubated with gentle shaking for 48 hours at 37°C. Culture is then analyzed (mobility, urease, catalase, and production of oxidase) and centrifuged (optionally after having grouped together several flasks) at 3,000 rpm for 20 minutes at 4°C. The pellet is resuspended in PBS (BioMérieux) containing 1% formalin (37% formalin,

Sigma). The volume is adjusted so as to obtain a final concentration of 2 mg/ml (1 ml having an OD of 1 at 600 nm before centrifugation corresponds to 377 μg of protein). The product is mixed gently at 4°C for 4 hours, washed 3 times in PBS, and the final solution is concentrated to 100 μg of protein/50 μl. The aliquots are kept at -70°C.

DC-chol liposomes containing urease are prepared as follows: first of all, to obtain a dry lipid film containing 100 mg of DC-chol (R-Gene Therapeutics) and 100 mg of DOPC (dioleylphosphatidylcholine) (Avanti Polar Lipids), these products are mixed in powdered form in about 5 ml of chloroform. The solution is allowed to evaporate under vacuum using a rotary evaporator. The film thus obtained on the walls of the container is dried under high vacuum for at least 4 hours. In parallel, 20 mg of a urease lyophilisate and 100 mg of sucrose are diluted in 13.33 ml of 20 mM Hepes buffer pH 7.2. Ten ml of this preparation (which contains 1.5 mg of urease and 0.75% sucrose) is filtered on the 0.220 μm Millex filter and then used to rehydrate the lipid film. The suspension is stirred for 4 hours and then either extruded (10 passes on a 0.2 μm polycarbonate membrane) or microfluidized (10 passes at a pressure of 500 kPa in a Microfluidics Co Y10 microfluidizer). In the liposome suspension thus obtained, the level of encapsulated urease is from 10 to 60%. This suspension is lyophilized after having adjusted the sucrose concentration to 5% (425 mg of sucrose are added per 10 ml). Before use, the lyophilisate is taken up in an appropriate volume of water or buffer and the suspension is purified on a discontinuous sucrose gradient (steps of 0, 30, and 60%) so as to obtain a preparation in which the quantity of encapsulated urease is greater than about 70% compared with the total quantity of urease.

Cholera toxin is used as mucosal adjuvant in an amount of 10 μg/dose of urease or of bacterial preparation.

The QS-21 (Cambridge Biosciences; Aquila) is used as adjuvant in an amount of 15 μg/dose of urease.

Challenge

Two weeks after the second booster, the mice were subjected to a gastric gavage with 300 μl of a suspension of a strain of *H. pylori* adapted to the mice, the strain ORV2002 (1×10^7 live bacteria in 200 μl of PBS; OD_{550} of about 0.5). One group which received no dose of antigen and which serves as control is challenged likewise.

Analysis of the challenge

Four weeks after the challenge, the mice were sacrificed by breaking the cervical vertebrae. The stomachs were removed in order to evaluate the urease activity and to make histological analyses. The urease activity was evaluated after 4 and 24 hours (OD at 550 nm) with the Jatrox test, Procter & Gamble) and after 24 hours the number of mice still negative was noted.

Measurement of the local antibody response by ELISPOT (salivary glands and stomach)

The ELISPOTs were performed in accordance with Mega *et al.*, J. Immunol. (1992) 148: 2030. The plates were coated with an extract of *H. pylori* proteins at a concentration of 50 μg/ml.

To test the antibody response at the level of the stomach, we modified the method as follows: half of the stomach was cut into 1-mm^2 pieces with an automatic apparatus for cutting human tissues (McIllwain Laboratories, Gilford, UK) and the digestion carried out with Dispase (2 mg/ml, Boehringer Mannheim) in 2 ml of a modified Joklil solution to which 10% horse serum (Gibco), glutamine and antibiotics were added. Four half-hour digestions were performed at 37°C with gentle mixing. The cells thus digested were filtered after each step using 70 μm filters (Falcon), and then washed 3 times in a solution of RPMI 1640 (Gibco) supplemented with 5% fetal calf serum (FCS), and incubated in the same solution for at least 4 hours in plates

covered with nitrocellulose (Millipore) (100 μl/well, 4 wells). Between 1 and 3 × 10^5 cells are obtained per half stomach (the cells of large size and the macrophages were not counted).

The biotinylated IgA and the streptavidin-biotinylated peroxidase complex were obtained from Amersham. The spots were revealed under the action of the AEC substrate (Sigma) and as soon as the plates are dry, they were counted under a microscope (magnification ×16 or ×40). The mean values corresponding to the number of IgA spots in four wells were calculated and expressed as the number of spots/10^6 cells.

Analysis of the response by ELISA

The analyses by ELISA were performed in accordance with the standard procedure (the biotinylated conjugates and the streptavidin-peroxidase were obtained from Amersham and the OPD (O-phenyl-diamine dihydrochloride) substrate from Sigma). The plates were coated with *H. pylori* extracts (5 μg/ml) in carbonate buffer. A control serum from mice directed against the *H. pylori* extract was introduced in each experiment. The titre corresponds to the reciprocal of the dilution giving an OD of 1.5 at 490 nm.

1B - Results

The results are presented in Figures 1 to 4 described above and by the following comments:

Figure 1 shows that when the subcutaneous route is used, much better results are obtained in terms of the local response both in the salivary glands and in the stomach if the administration took place in the posterior part of the mice, that is to say in the sublumbar region.

Before any comment on the subject of Figures 2 to 4, it should be noted that these figures present the results obtained with the antigen used in the form with

cholera toxin adjuvant and administered by the intragastric route. This experiment is termed standard reference experiment since the prior art CT/IG combination is that which gives the best results up until now.

Figure 2 compares the results obtained with a preparation of inactivated bacteria without adjuvant, by the intragastric route and subcutaneous route. It is clear that much better results are obtained when the subcutaneous route is used while targeting the sublumbar region. Furthermore, the results obtained after administration by the subcutaneous route are identical to, if not slightly better than, those which are obtained in the standard reference experiment with the same preparation, this time with the cholera toxin adjuvant and administered by the intragastric route.

Furthermore, reference can be made to experiments (a) to (e) the results of which in terms of urease activity 4 hours after the mice have been sacrificed are reported in Figure 2 and it is indicated that the number of mice which are still negative for the urease activity 24 hours after having been sacrificed is respectively (a) 0/8, (b) 4/8, (c) 4/8, (d) 0/8, and (e) 10/10. This is in agreement with what was previously concluded in the paragraph; namely that experiment (b) leads to results similar to those obtained during the standard reference experiment.

Figure 3 shows that a urease preparation encapsulated into DC-chol liposomes and administered by the subcutaneous route in the sublumbar region gives results as good as those obtained in the standard reference experiment.

Furthermore, reference can be made to experiments (a) to (d) whose results in terms of urease activity 4 hours after the mice have been sacrificed are reported in Figure 3 and it is indicated that the number of mice which are still negative for the urease activity 24 hours after having been sacrificed is respectively (a) 5/10, (b) 4/10, (c) 0/10, and (d) 10/10. This is in agreement with what was concluded in the preceding paragraph; namely that experiment (a) leads to results similar to those obtained during the standard reference experiment.

Figure 4 shows that a urease preparation with QS-21 adjuvant and administered

by the subcutaneous route in the sublumbar region gives results as good as those obtained in the standard reference experiment.

Furthermore, reference can be made to experiments (a) to (d) whose results in terms of urease activity 4 hours after the mice have been sacrificed are reported in Figure 4 and it is indicated that the number of mice which are still negative for the urease activity 24 hours after having been sacrificed is respectively (a) 1/8, (b) 5/8, (c) 0/8, and (d) 10/10. This is in agreement with what was concluded in the preceding paragraph; namely that experiment (b) leads to results similar to those obtained during the standard reference experiment.

The table below presents the quantities of IgA, IgG1, and IgG2a induced during experiments whose results in terms of urease activity are reported in Figures 2 to 4 as well as the number of mice whose urease activity is characterized by an OD of less than 0.1 after 4 and 24 hours after sacrifice. The quantities of IgA, IgG1, and IgG2a are expressed as ELISA titre.

	urease[1] CT IG	WC IG	WC CT IG	WC SC	urease lipo DC-chol SC	urease QS-21 SC
IgA	45	91	107	63	0	1
IgG1	65700	1920	349	1273146	620000	2970399
IgG2a	20200	399	3440	42900	321000	1136095
OD < 0.1 4 hours	5/10	0/8	5/8	6/8	5/10	6/8
OD < 0.1 24 hours	4/10	0/8	4/8	4/8	5/10	5/8

Example 2: Immunization studies in monkeys

2A - Materials and methods

Monkeys

Twenty eight 2-year old monkeys (*Macaca fascicularis*) obtained from Mauritius were used in this study. Before subjecting the monkeys to the various immunization procedures described below, a biopsy showed that most of them were

chronically infected with organisms similar to *Gastrospirillum hominis* (GHLO) or *H. heilmanii*.

Administration procedures

Since nearly all the monkeys were infected with GHLOs, it was decided to test the efficacy of the various procedures in therapy. Three procedures were used, as summarized in the table below:

Group	D0	D21	D42	D63
1 and 1u	IN + IG	IN + IG	IN + IG	IN + IG
2 and 2u	IM	IM	IM	IM
3 and 3u	IM	IN + IG	IM	IN + IG

It is specified that the administration by the intramuscular route was carried out in the dorsolumbar muscles.

Antigens and adjuvants

Since there is a cross-reactivity between the GPLOs and *H. pylori*, it was chosen to use a preparation of inactivated *H. pylori* bacteria, as described in Example 1A, alone or in combination with recombinant urease prepared according to the method referenced in Example 1A.

The *E. coli* heat-labile toxin (LT) (Sigma) or the B subunit of the cholera toxin (CTB) (Pasteur Mérieux sérums & vaccins) was used as mucosal adjuvant whereas DC-chol was used as parenteral adjuvant. DC-chol powder is simply rehydrated with an antigen preparation.

The doses used are as follows:

Route	Microorganisms	Urease	DC-chol	LT	CTB
IG	400 μg	2.5 mg	-	25 μg	-
IN	400 μg	400 μg	-	25 ng	25 μg
IM	400 μg	100 μg	400 μg	-	-

Biopsies, urease test, and bacteriological/histological study

A biopsy was performed on each of the monkeys before and after immunization (one month after the third booster). Using the biopsies, a urease test and a histological study were performed.

The urease activity is evaluated using the Jatrox kit (Procter & Gamble). The level of this activity is estimated as follows, in a decreasing manner: level 3, pink color appearing during the first 10 minutes; level 2, pink color appearing between 10 and 30 minutes after the addition of the reagents; level 1, pink color appearing between 30 minutes and 4 hours and level 0, weak or no color after 4 hours.

The histological studies were performed using biopsies fixed in formalin and the bacterial load was quantified as follows: absence of bacteria (0); a few bacteria of the *Helicobacter* type (0.5); fairly numerous bacteria (1); numerous bacteria (2); highly numerous bacteria (3). A difference of one level (for example from 1 to 2) corresponds to a number of bacteria 5 times greater.

Analysis of the response by the ELISA test

An ELISA test is carried out as described in Example 1A.

2B - Results

The table below relates to the bacterial load which, before and after immunization, is assessed using two tests: (i) by evaluating the urease activity and (ii) by carrying out a histological study. The results relating thereto are presented in columns 3 to 6. The last three columns indicate for each group (control, 1, 2, or 3) the

number of monkeys for which the bacterial load remains unchanged after immunization (→) according to the two tests; or appears lower (↘) or increased (↗) in at least one of the two tests, the other test indicating a stationary bacterial load. When the results of the two tests show a similar variation, the upwards or downwards arrow is double.

Monkeys	Group	Urease activity before immunization	Urease activity after immunization	Histology before immunization	Histology after immunization	Variation ↘	↗	↘
H 282	C	2-2	3-2	2	3-2			
J 005	C	2-2	2-1	2	1-0	1/4	1/4	2/4
J 852	C	0-0	2-0	0	1-1			(2/4↗↗)
J 476	C	0-0	2-0	0	1-1			
H 799	1	2-2	2-2	2	2-2			
J 845	1	2-2	3-2	2	2-1			
J 205	1	1-1	2-2	0	1			
J 328	1	2-2	1-2	3	3-2	1/8	5/8	2/8
J 197	1u	2-2	3-2	2	3			(1/8↗↗)
H 025	1u	2-2	2-2	1	1-1			
G 460	1u	2-2	3-2	3	2-3			
J 607	1u	2-2	2-2	2	2			
H 549	2	3-3	2-2	3	2-3			
H 622	2	3-3	1-1	2	2-3			
H 504	2	3-3	1-1	2	2-1			
H 798	2	1-1	0-1	1	1-1			
J 367	2u	2-2	2-1	3	2-3	6/8	1/8	1/8
G 486	2u	2-2	2-2	1	2-2			
J 522	2u	2-2	0-0	2	2-2			
G 722	2u	3-3	2-0	2	2-3			
H 820	3	3-3	2-2	3	2-2			
J 557	3	2-2	1-0	2	1-2*			
H 588	3	2-2	2-0	3	1-2			
J 153	3	3-3	3-3	2	3-3	5/8	0	3/8
H 480	3u	2-2	2-2	2	3-3	(3/8↘↘)		
J 344	3u	3-3	2-0	3	2-2			
H 710	3u	2-2	2-2	2	3-3			
J 262	3u	3-3	2-2	3	3-2			

Thus, this table reveals that in the group having been subjected to an immunization procedure by the strict mucosal route, the results are substantially identical to those obtained with the negative control group. On the other hand, in the groups having been subjected to an immunization procedure by the mixed mucosal and intramuscular route or by the strict intramuscular route, a marked reduction in the bacterial load is observed. This highlights the importance of the immunization conditions and in particular of the immunization rate; consequently, the use of an immunization procedure which employs the parenteral route targeted in the subdiaphragmatic region, is recommended in order to obtain a protective effect.

These results are to be placed in perspective with other results relating to the serum antibody levels which are presented in Figure 3. This figure shows that the immunization scheme by the strict mucosal route (1 and 1u) leads to results which are very similar to those of the negative control group. On the other hand, the immunization scheme by the mixed mucosal and intramuscular route (2 and 2u), and better still the immunization scheme by the strict intramuscular route (3 and 3u), makes it possible to induce antibody levels substantially greater than those of the control group.

Thus, a high serum response may be correlated with a protective effect, whereas *a contrario*, a low response is linked to the absence of a protective effect. The immunization conditions which make it possible to obtain the desired effect (high serum response and protective effect) include the use of the parenteral route targeted in the subdiaphragmatic region or that of a Th1 adjuvant.

Example 3: Treatment of an *H. pylori* infection in mice

We compared the efficacy of immunization *via* the subcutaneous (SC) route with that of the mucosal route, in order to treat an *H. pylori* infection in a mouse model.

OF1 mice were infected with 10^6 colony-forming units (cfu) of the *H. pylori*

strain ORV2001. After one month, verification that the infection was well-established was made by randomly sacrificing 10/100 mice and testing the urease activity on a quarter of the entire stomach. Since all of the results were positive, the mice were then immunized (10 per group) 3 times at 3 weekly intervals, either subcutaneously using 10 μg of recombinant urease supplemented with 15 μg of QS-21 (Aquila) or 400 μg of adjuvant Bay R1005 (Bayer), or orally using 40 μg of urease mixed with 1 μg of LT. For each of the two adjuvants administered parenterally, the immunization was carried out either in the neck, in order to reach the lymphatic ganglions of the upper region of the body, or in the lumbar region, in order to reach the abdominal lymphatic ganglions. Ten mice were left uninfected and unimmunized (negative control), whereas the mice of the positive control received a saline solution, QS-21, or Bay adjuvant subcutaneously (lumbar region).

One month after the third immunization, all of the mice were sacrificed and the stomachs removed to evaluate the extent of the colonization by measuring the urease activity (10/10 mice were analyzed in each group), as well as by carrying out quantitative culturing (5/10 were analyzed). Figures 6A (test relating to urease) and 6B (culturing) show that in the mice immunized with urease supplemented with QS-21, subcutaneously in the lumbar region, the infection had virtually disappeared (4/5 mice were negative in quantitative culturing). The mice immunized with urease subcutaneously in the neck, in the presence of QS-21, and the mice that received urease plus LT orally exhibited a 10- to 100-fold decrease in the infection when compared with the unimmunized mice. The Bay adjuvant induced an identical decrease, which was more pronounced in the mice immunized in the lumbar region.

Histopathology carried out on these same mice did not reveal any gastritis that was more extensive than in the controls.

As we observed in our previous prophylactic study (Example 1), the protected mice had a high level of the two isotypes IgG1 and IgG2 in the serum, which is representative of a Th2/Th1 equilibrated response. Furthermore, the mice immunized

subcutaneously in the lumbar region had the highest levels of IgA in the serum, which demonstrates a mucosal response.

These results show that targeted, systemic immunization is capable of curing an acquired *H. pylori* infection in mice, and that the use of adjuvants that induce an equilibrated mucosal response of Th1/Th2 type is desirable in order to achieve this aim.

Example 4: Mucosal prime/parenteral boost strategy for urease immunization that elicits protection in mice against infection with *H. pylori*

Swiss Webster mice were immunized with a mucosal prime/parenteral boost strategy. A single oral dose of 25 μg recombinant *H. pylori* urease (urease) and 5 μg *Escherichia coli* heat labile enterotoxin (LT) was administered as a prime. Three weeks later, mice were boosted by the parenteral route with 2 doses, 3 weeks apart, with 100 μg urease + 100 μg alum. Mice immunized by this prime/boost strategy exhibited a 2,000-fold reduction in the median *H. pylori* colony forming units (CFUs) compared to unimmunized controls (Figure 7). Immunization by this strategy was more efficacious than 3 doses of mucosal vaccine when a 1,000-fold reduction in colonization was achieved. A single priming dose of orally administered urease + LT only produced a 10-fold reduction in median *H. pylori* CFUs, boosting with urease administered parenterally without an adjuvant resulted in only a 2-fold reduction in medium CFUs (Figure 7).

This immunization strategy was used to immunize rhesus monkeys against challenge with *H. pylori*.

Example 5: Protection of rhesus monkeys from *H. pylori* infection by urease immunization using a mucosal prime/parenteral boost strategy

Rhesus monkeys, seronegative for *H. pylori*, were treated with quadruple therapy and confirmed to be free of *H. pylori* infection by culture and histologic

examination of gastric biopsies. Nineteen monkeys were randomized into 4 groups: 4 monkeys were sham vaccinated, 5 received recombinant *H. pylori* urease (urease) vaccine given by the intramuscular route with alum (aluminum hydroxide, Rehydragel) as an adjuvant, 5 received a mucosal priming dose of urease vaccine given by the oral route with *Escherichia coli* heat labile enterotoxin (LT) as an adjuvant, followed by two parenteral boosts of urease vaccine given by the intramuscular route with alum, and 5 monkeys received urease vaccine given by the intramuscular route with Bay adjuvant. Monkeys were immunized every three weeks for the first three immunizations, and a fourth dose was administered at 20 weeks.

One week after the last immunization, the monkeys were challenged with *H. pylori*. The monkeys were sacrificed 10 weeks after challenge. At necropsy, 10 gastric sites (1 cardiac, 2 corporal, 3 corporal-antral junction, 3 antral, and 1 pyloric) were sampled for bacterial culture by taking 5 mm diameter punch biopsies. Additional tissues from the 5 regions of the stomach were harvested for histopathology.

Although all of the monkeys were infected with *H. pylori* as a result of the experimental challenge, monkeys immunized with urease + LT as a mucosal prime followed by 3 parenteral booster doses of urease + alum showed statistically significant reduction ($p=0.05$, Wilcoxon rank sums test) in colonization when compared to the control, sham immunized monkeys (Figure 8). A greater than 20-fold reduction in median bacterial colony forming units per bunch biopsy (5.8×10^2 CFU, ranging from 1×10^2 to 6.7×10^2 CFU) was found in monkeys that received the vaccine as a mucosal prime/parenteral boost regimen, compared to a median CFU of 1.3×10^4 (range 1.5×10^3 to 1.8×10^5 CFU) for the group of sham immunized monkeys.

The group of monkeys receiving urease vaccine in a mucosal prime/parenteral boost regimen had similar gastritis (Figure 9) and epithelial changes (Figure 10) after challenge with *H. pylori*. There was no evidence that vaccination enhanced either

gastritis or epithelial alterations.

In contrast to the monkeys receiving the mucosal prime/parenteral boost regimen, monkeys immunized by the parenteral route with urease + Bay showed no difference in *H. pylori* colonization compared with the sham-immunized controls ($p = 1.00$), while monkeys treated with urease + alum showed a partial effect ($p=0.33$) (Figure 8). Culture data was unavailable for one of the monkeys in the group receiving urease + Bay, due to heavy contamination of gastric samples with other bacteria.

These results show that an immunization scheme utilizing a mucosal prime of urease + LT followed by parenteral boosting with urease + alum is efficacious in preventing *H. pylori* infection in non-human primates. This scheme was chosen with the rationale that the immune system must be primed to respond to a mucosal infection, *i.e.*, by mucosal immunization with an appropriate adjuvant. However, once the immune response is 'set' properly, a more conventional adjuvant, such as alum, can be used as a parenteral immunization to boost the response. This kind of scheme is not only more effective than a mucosal only regimen, but it utilizes less antigen because the parenteral antigen dose (100 μg urease) can be much less than a mucosal dose (4 mg).

Other embodiments are within the following claims.

Claims

1. Use of an immunogenic agent derived from *Helicobacter*, in the manufacture of a pharmaceutical composition intended for the induction of a T helper 1 (Th1) type immune response against *Helicobacter*, to prevent or treat a *Helicobacter* infection in a mammal.

2. Use according to Claim 1, in which the Th1-type immune response is characterized either (i) by a ratio of the ELISA IgG2a:IgG1 titers in mice greater than or equal to 1:100, or (ii) by a ratio of the ELISA IgG2a:IgA titers in mice greater than or equal to 1:100.

3. Use according to Claim 2, in which the Th1-type immune response is characterized either (i) by a ratio of the ELISA IgG2a:IgG1 titers in mice greater than or equal to 1:10, or (ii) by a ratio of the ELISA IgG2a:IgA titers in mice greater than or equal to 1:10.

4. Use according to Claim 3, in which the Th1-type immune response is characterized either (i) by a ratio of the ELISA IgG2a:IgG1 titers in mice greater than or equal to 1:2, or (ii) by a ratio of the ELISA IgG2a:IgA titers in mice greater than or equal to 1:2.

5. Use of an immunogenic agent derived from *Helicobacter*, in the manufacture of a pharmaceutical composition intended to be administered by the systemic route, in the part of a mammal, especially the primate, situated under its diaphragm, to treat or prevent a *Helicobacter* infection.

6. Use according to Claim 5, in which the composition is capable of inducing a Th1-type immune response when it is administered by the subdiaphragmatic systemic

route.

7. Use according to Claim 5 or 6, in which the Th1-type immune response is characterized either (i) by a ratio of the ELISA IgG2a: IgG1 titers greater than or equal to 1:100, or (ii) by a ratio of the ELISA IgG2a:IgA titers greater than or equal to 1 : 100.

8. Use according to Claim 7, in which the Th1-type immune response is characterized either (i) by a ratio of the ELISA IgG2a:IgG1 titers in mice greater than or equal to 1:10, or (ii) by a ratio of the ELISA IgG2a:IgA titers in mice greater than or equal to 1:10.

9. Use according to Claim 8, in which the Th1-type immune response is characterized either (i) by a ratio of the ELISA IgG2a:IgG1 titers in mice greater than or equal to 1:2, or (ii) by a ratio of the ELISA IgG2a:IgA titers in mice greater than or equal to 1:2.

10. Use according to one of Claims 1 to 9, in which the immunogenic agent derived from *Helicobacter* is selected from a preparation of inactivated *Helicobacter* bacteria, a *Helicobacter* cell lysate, a peptide, a polypeptide from *Helicobacter* in purified form, a DNA molecule comprising a sequence encoding a peptide or a polypeptide from *Helicobacter* placed under the control of the elements necessary for its expression and a vaccinal vector comprising a sequence encoding a peptide or a polypeptide from *Helicobacter* placed under the control of the elements necessary for its expression.

11. Use according to Claim 10, in which the immunogenic agent derived from *Helicobacter* is the UreB or UreA subunit of the *Helicobacter* urease.

12. Use according to Claim 10, in which the immunogenic agent derived from *Helicobacter* is a DNA molecule or a vaccinal vector comprising a sequence encoding the UreB or UreA subunit of the *Helicobacter* urease.

13. Use according to Claim 10, 11, or 12, in which the immunogenic agent is derived from *Helicobacter pylori*.

14. Use according to one of Claims 5 to 13, in which the pharmaceutical composition is intended to be administered by the strict systemic route.

15. Use according to one of Claims 5 to 14, in which the pharmaceutical composition is intended to be administered by a systemic route selected from the subcutaneous route, the intramuscular route, and the intradermal route.

16. Use according to one of Claims 5 to 14, in which the pharmaceutical composition is intended to be administered by a mucosal route followed by a parenteral route.

17. Use according to Claim 16, in which the pharmaceutical composition is intended to be administered by a parenteral route, followed by a mucosal route, followed by a parenteral route, followed by a mucosal route.

18. Use according to one of Claims 5 to 17, in which the pharmaceutical composition is intended to be administered in the dorsolumbar region of the said mammal.

19. Use according to one of Claims 5 to 18, in which the pharmaceutical composition is intended to be administered twice or three times by the systemic route

during the same treatment, in order to prevent or treat a *Helicobacter* infection.

20. Use according to one of Claims 5 to 18, in which the immunogenic agent is selected from a preparation of inactivated *Helicobacter* bacteria, a *Helicobacter* cell lysate, a peptide, a polypeptide from *Helicobacter* in purified form and is, in addition, combined with at least one compound capable of promoting the induction of a Th1-type immune response.

21. Use according to Claim 20, in which the compound capable of promoting the induction of a Th1-type immune response is selected from liposomes, microspheres, QS-21, DC-chol, and Bay R1005.

22. Use according to Claim 20, in which the compound capable of promoting the induction of a Th1-type immune response is selected from QS-21, DC-chol, and Bay R1005.

23. Use according to Claim 22, in which the immunogenic agent is combined with at least two compounds capable of promoting the induction of a Th1-type immune response; the first compound being selected from liposomes, microspheres and the second compound being selected from QS-21, DC-chol, and their equivalents.

24. Use according to Claim 20, in which the immunogenic agent is a peptide or a polypeptide which is combined, by covalent bonding, with at least one lipid capable of promoting the induction of a Th1-type immune response, so as to form a lipopeptide or lipid-containing polypeptide conjugate.

25. A method of preventing or treating *Helicobacter* infection in a mammal, said method comprising in order the steps of:

mucosally administering an immunogenic agent derived from *Helicobacter* to said mammal; and then

parenterally administering said immunogenic agent derived from *Helicobacter* to said mammal.

26. The method of claim 25, in which more than one mucosal administration is carried out.

27. The method of claim 25, in which more than one parenteral administration is carried out.

28. The method of Claim 25, in which the mucosal administration is carried out to prime an immune response to said immunogenic agent derived from *Helicobacter*, and the parenteral administration is carried out to boost an immune response to said immunogenic agent derived from *Helicobacter*.

29. The method of Claim 25 or 28, in which the mucosal administration is oral administration.

30. The method of Claim 25 or 28, in which the parenteral administration is intramuscular administration or subcutaneous administration.

31. The method of Claim 25, in which the immunogenic agent derived from *Helicobacter* is selected from a preparation of inactivated *Helicobacter* bacteria, a *Helicobacter* cell lysate, a peptide, a polypeptide from *Helicobacter* in purified form, a DNA molecule comprising a sequence encoding a peptide or a polypeptide from *Helicobacter* placed under the control of the elements necessary for its expression and a vaccinal vector comprising a sequence encoding a peptide or a polypeptide from

Helicobacter placed under the control of the elements necessary for its expression.

32. The method of Claim 31, in which the immunogenic agent derived from *Helicobacter* is the UreB or UreA subunit of the *Helicobacter* urease.

33. The method of Claim 31, in which the immunogenic agent derived from *Helicobacter* is a DNA molecule or a vaccinal vector comprising a sequence encoding the UreB or UreA subunit of the *Helicobacter* urease.

34. The method of Claim 31, 32, or 33, in which the immunogenic agent is derived from *Helicobacter pylori*.

35. The method of Claim 25, in which a mucosal adjuvant selected from the group consisting of *Escherichia coli* heat labile enterotoxin (LT), cholera toxin (CT), *Clostridium difficile* toxin, *Pertussis* toxin (PT), and combinations, subunits, toxoids, and mutants derived therefrom, is co-administered with the mucosally administered immunogenic agent.

36. The method of Claim 25, in which a parenteral adjuvant selected from the group consisting of alum, QS-21, DC-chol, and Bay is co-administered with the parenterally administered immunogenic agent.

Figure 1A

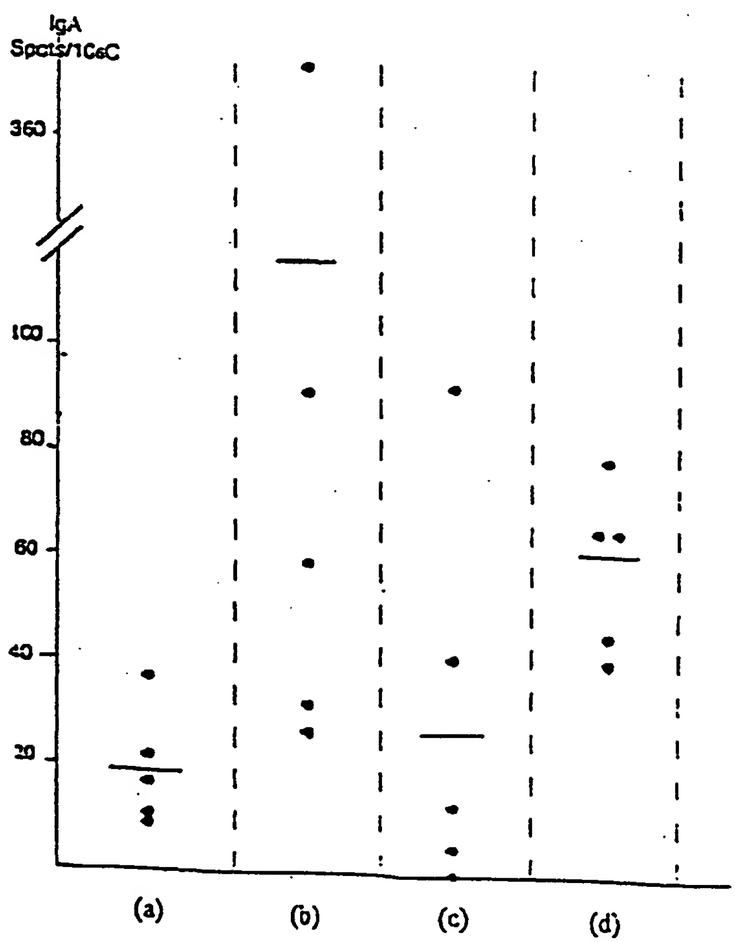


Figure 13

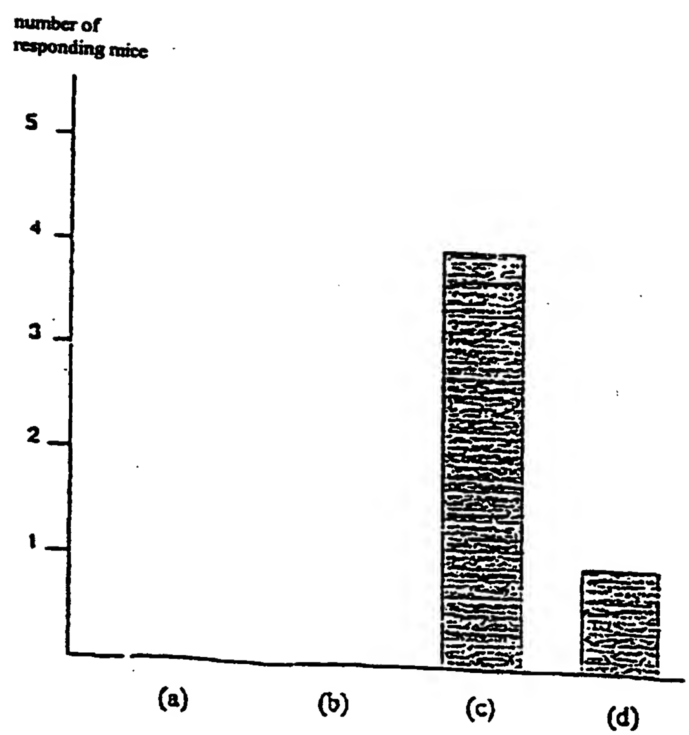


Figure 2

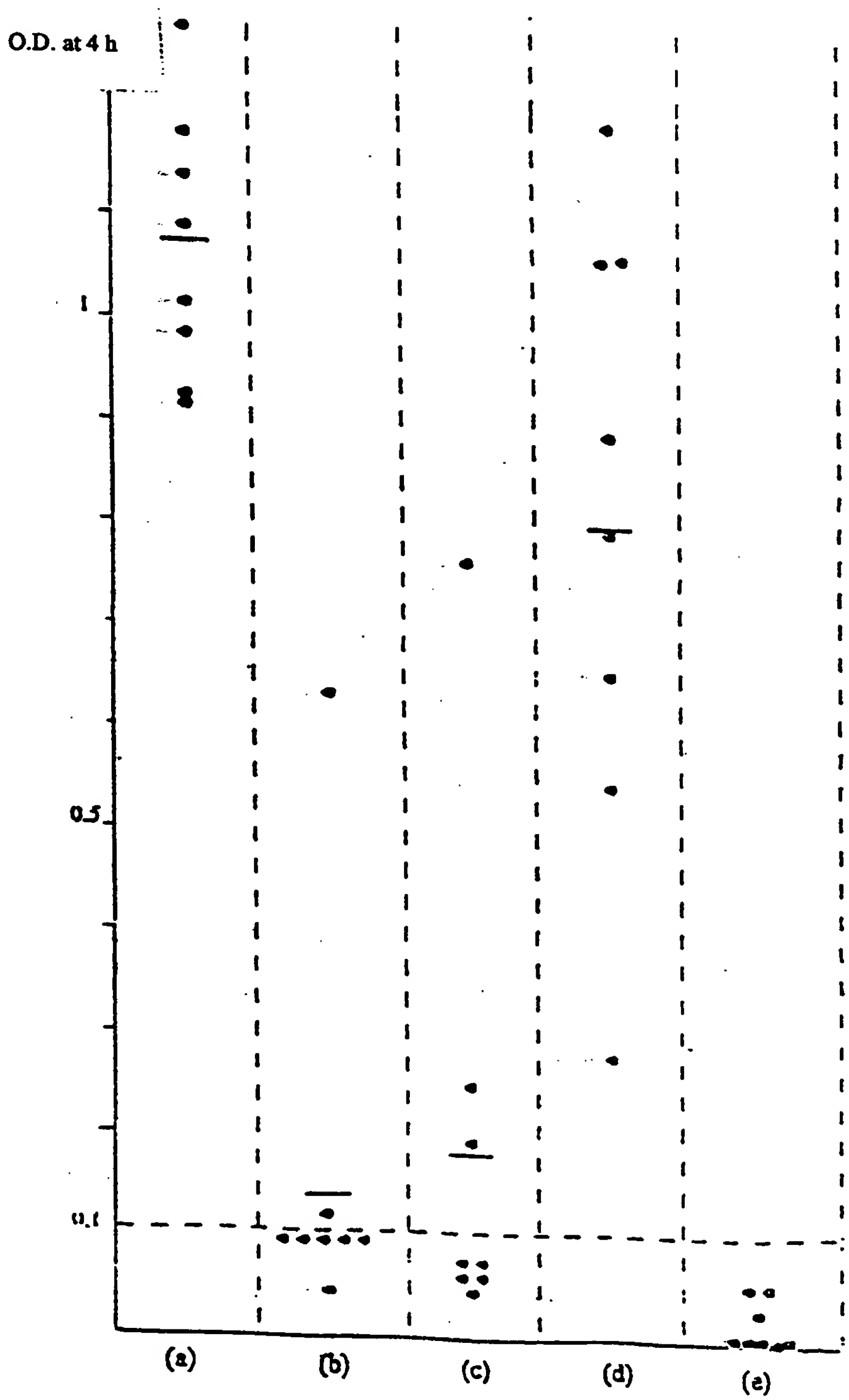


Figure 3

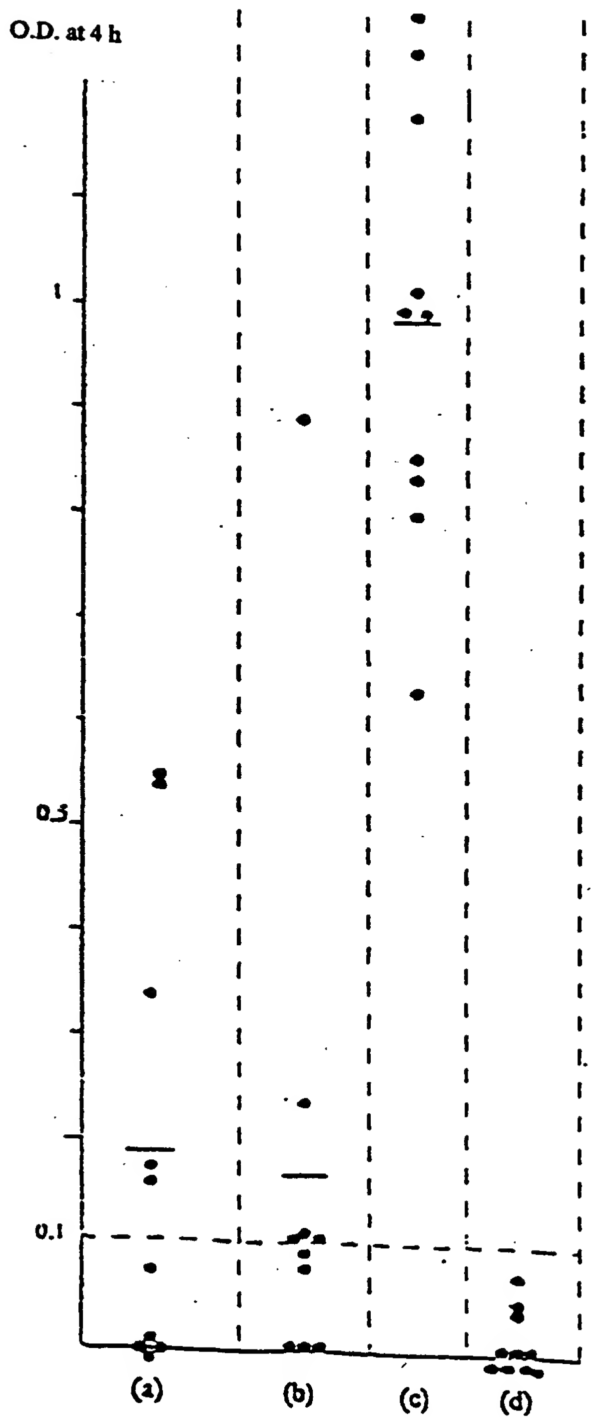


Figure 4

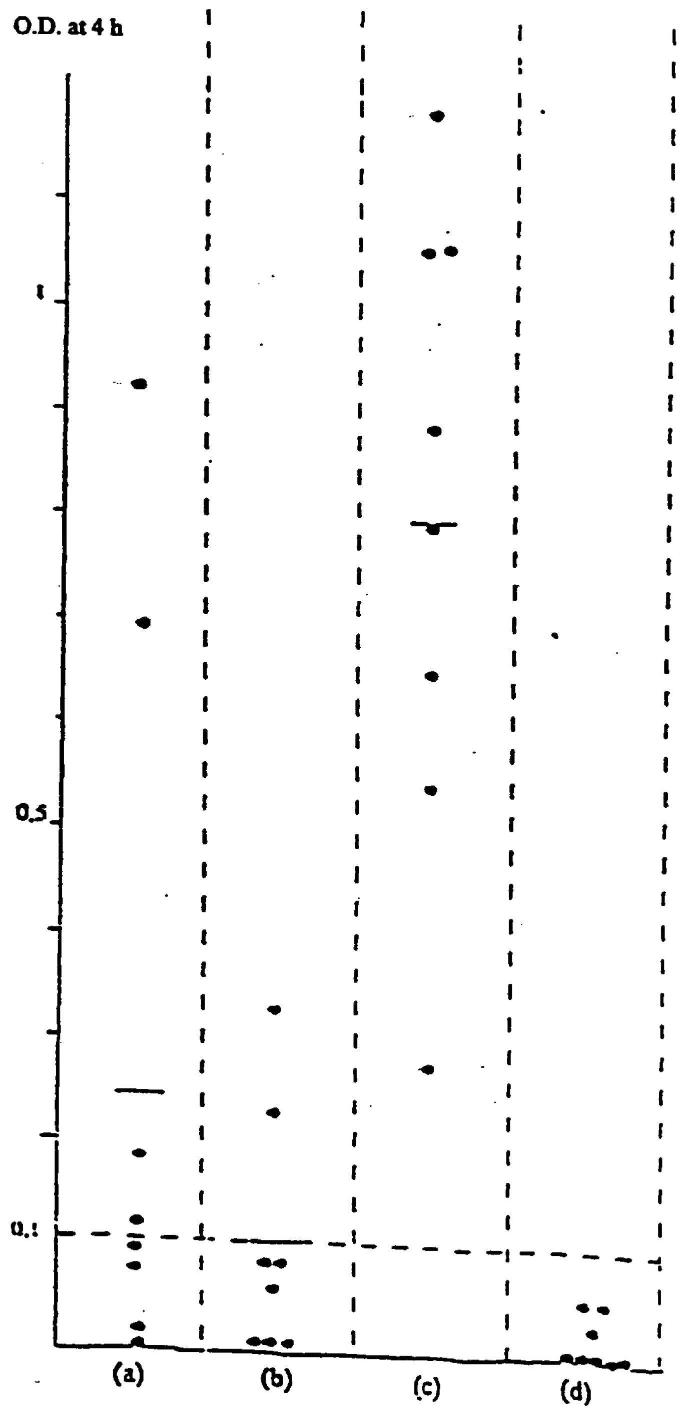
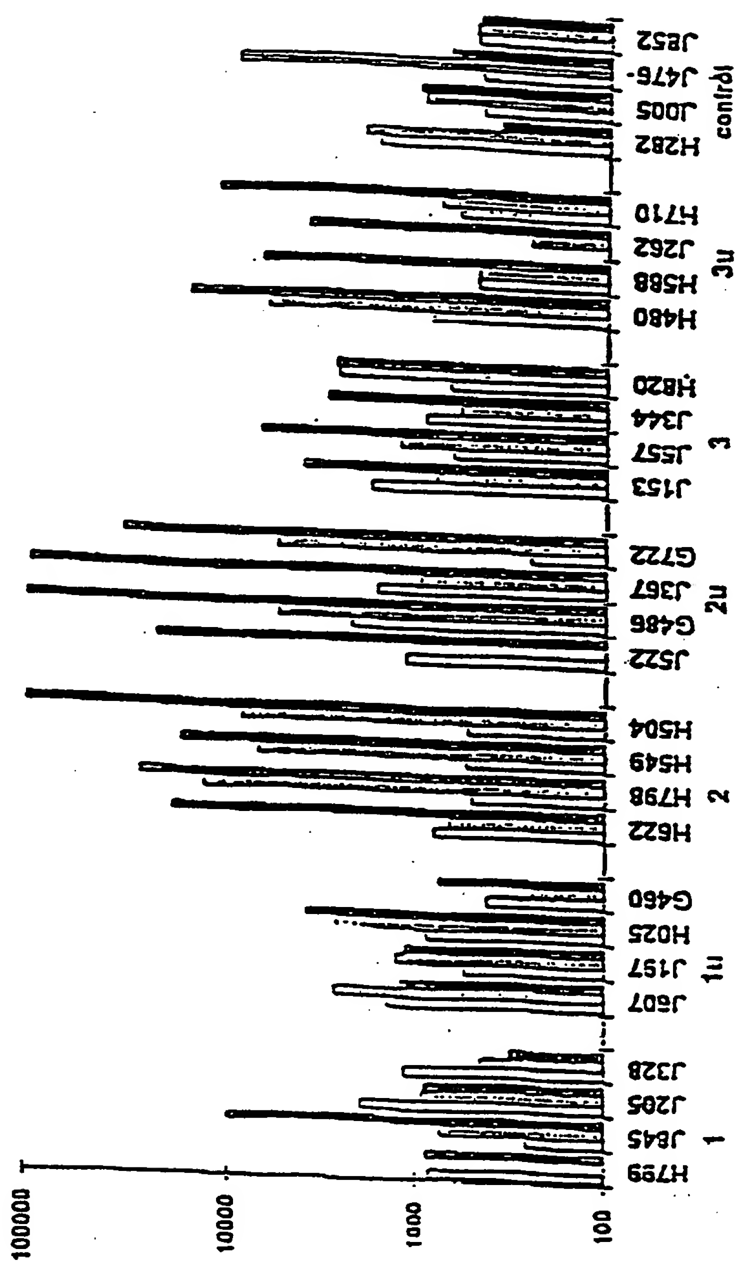


Figure 5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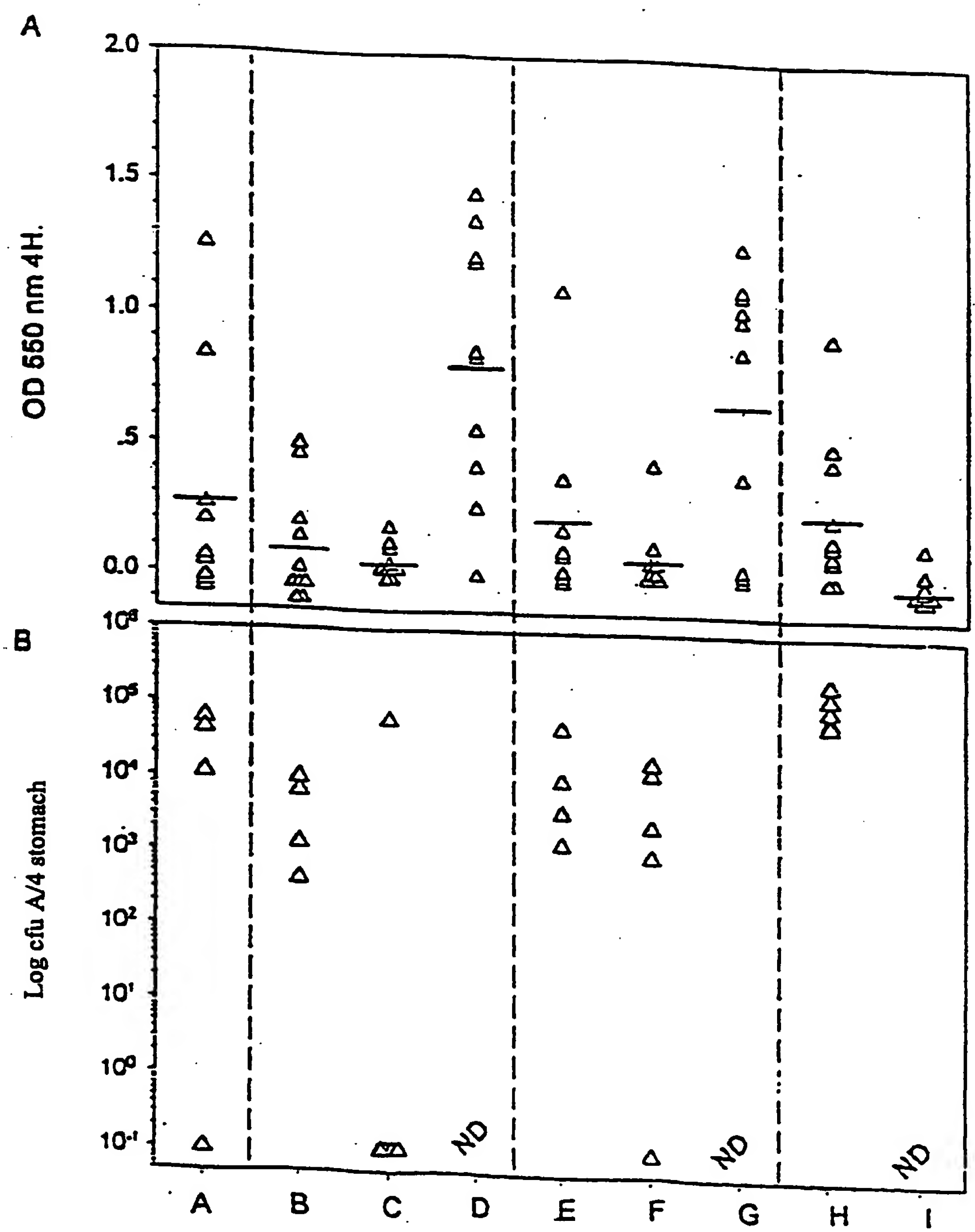
10000
1000
100
H799
J845
J205
J328
J507
J157
H025
G460
H622
H798
H549
H504
J522
G486
J367
G722
J153
J557
J344
H820
H480
H588
J262
H710
H282
J005
J476
J852
1
1u
2
2u
3
3u
control

Figures 6A and 6B



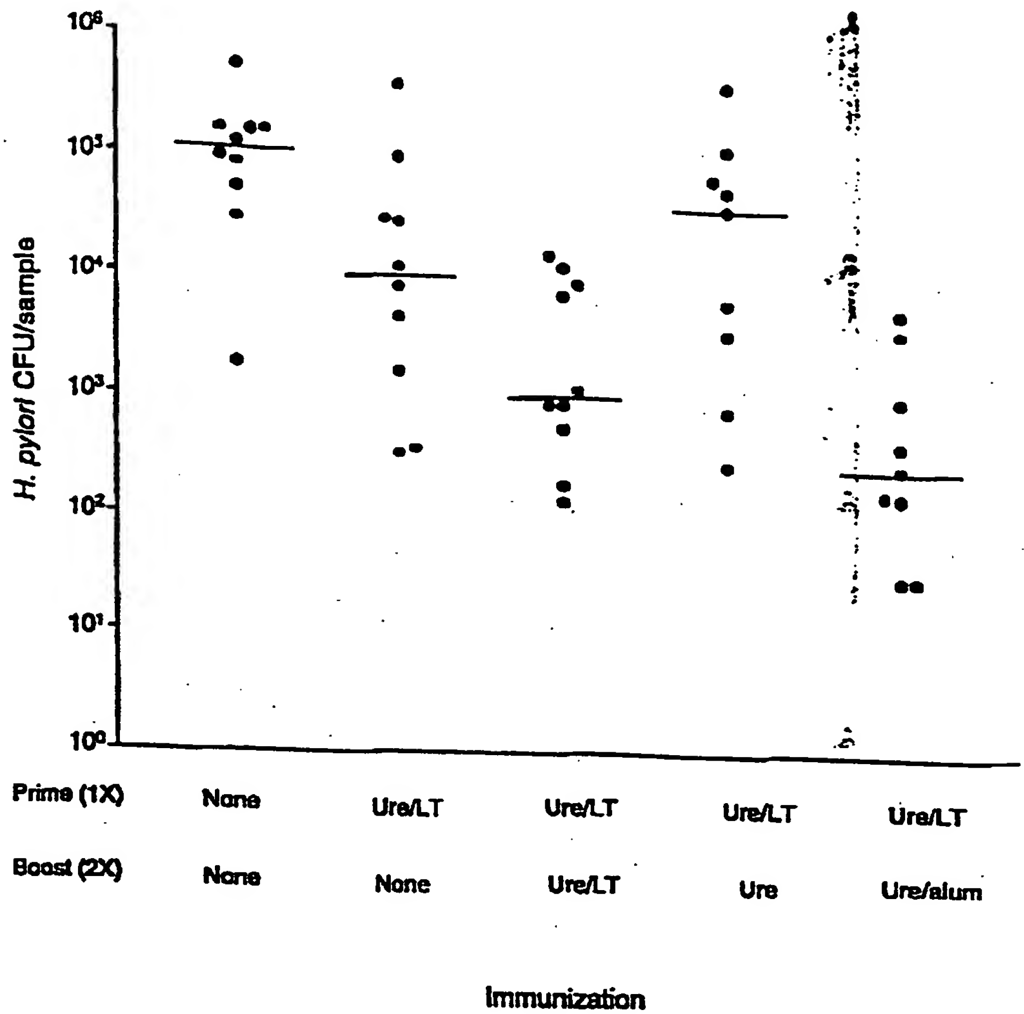

10⁶
10⁵
10⁴
10³
10²
10¹
10⁰
H. pylori CFU/sample
Prime (1X)
None
Ure/LT
Ure/LT
Ure/LT
Ure/LT
Boost (2X)
None
None
Ure/LT
Ure
Ure/alum
Immunization

Figure 7

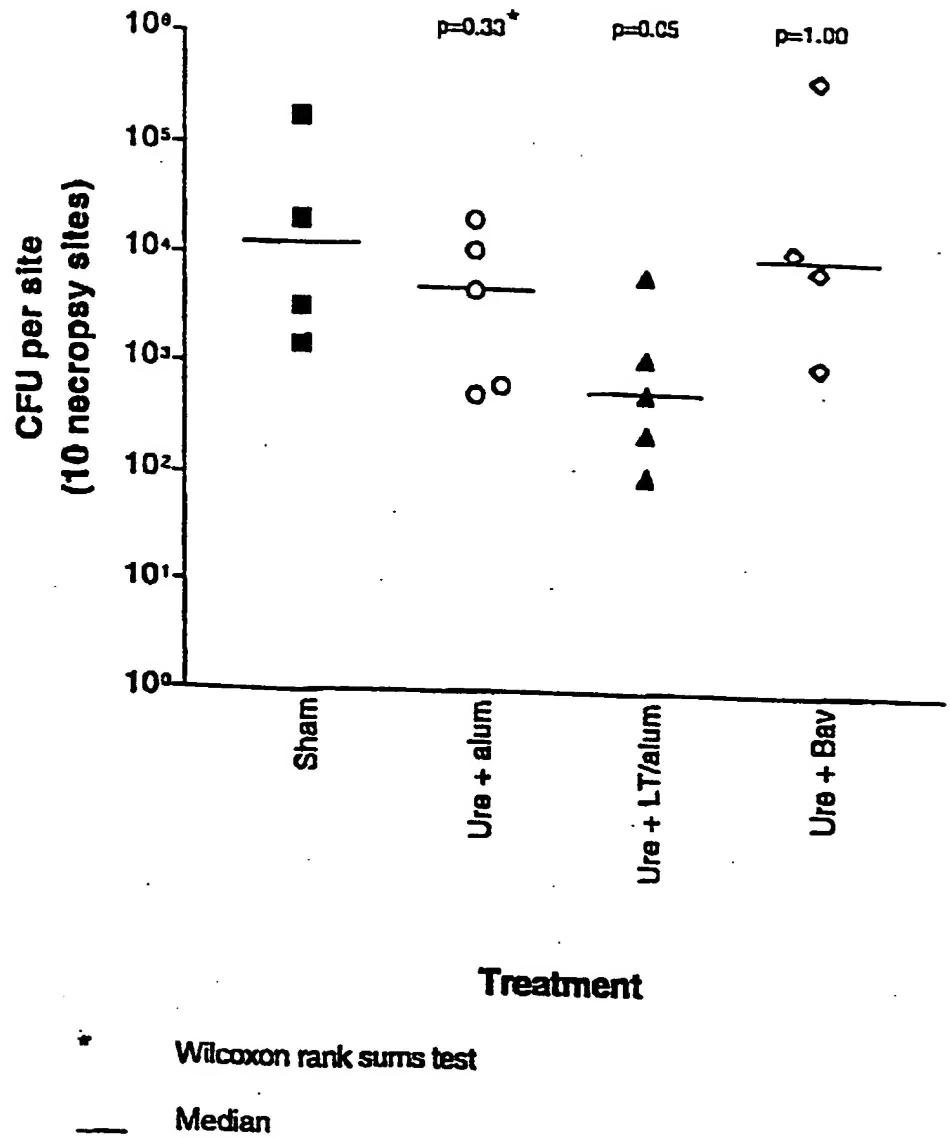

p=0.33*
p=0.05
p=1.00
CFU per site
(10 necropsy sites)
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Sham
Ure + alum
Ure + LT/alum
Ure + Bav
Treatment
* Wilcoxon rank sums test
Median

Figure 8

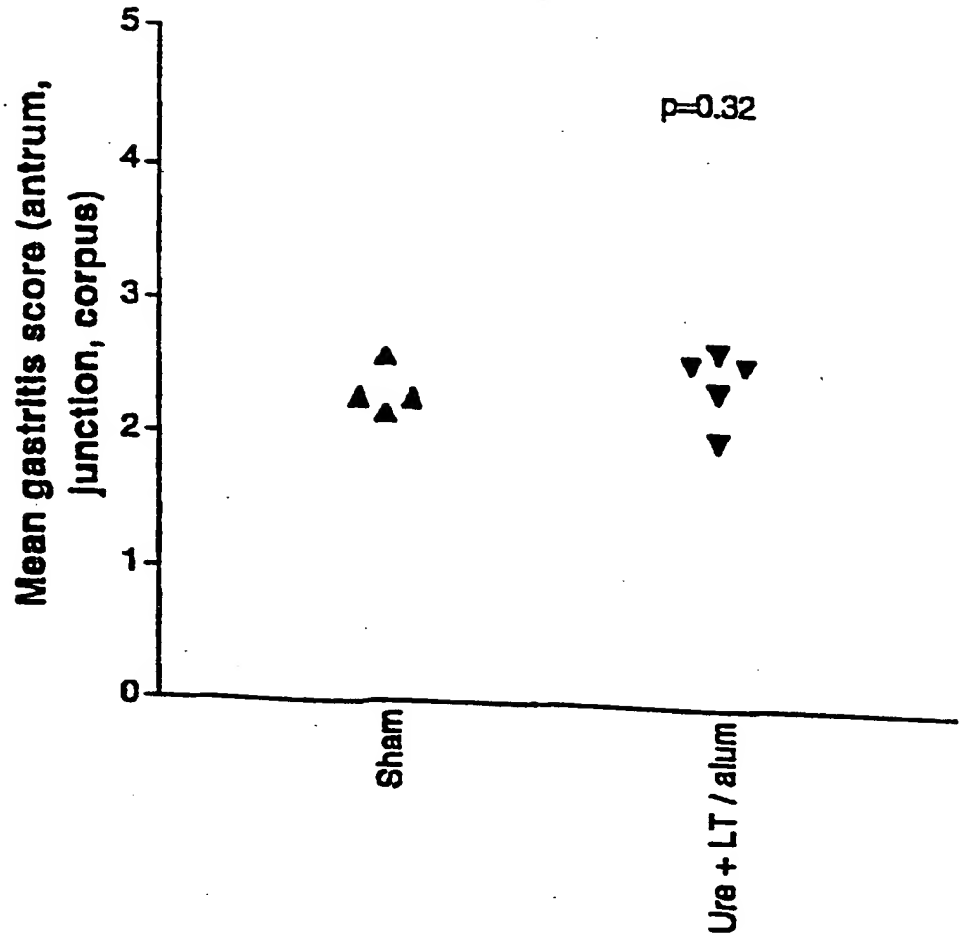
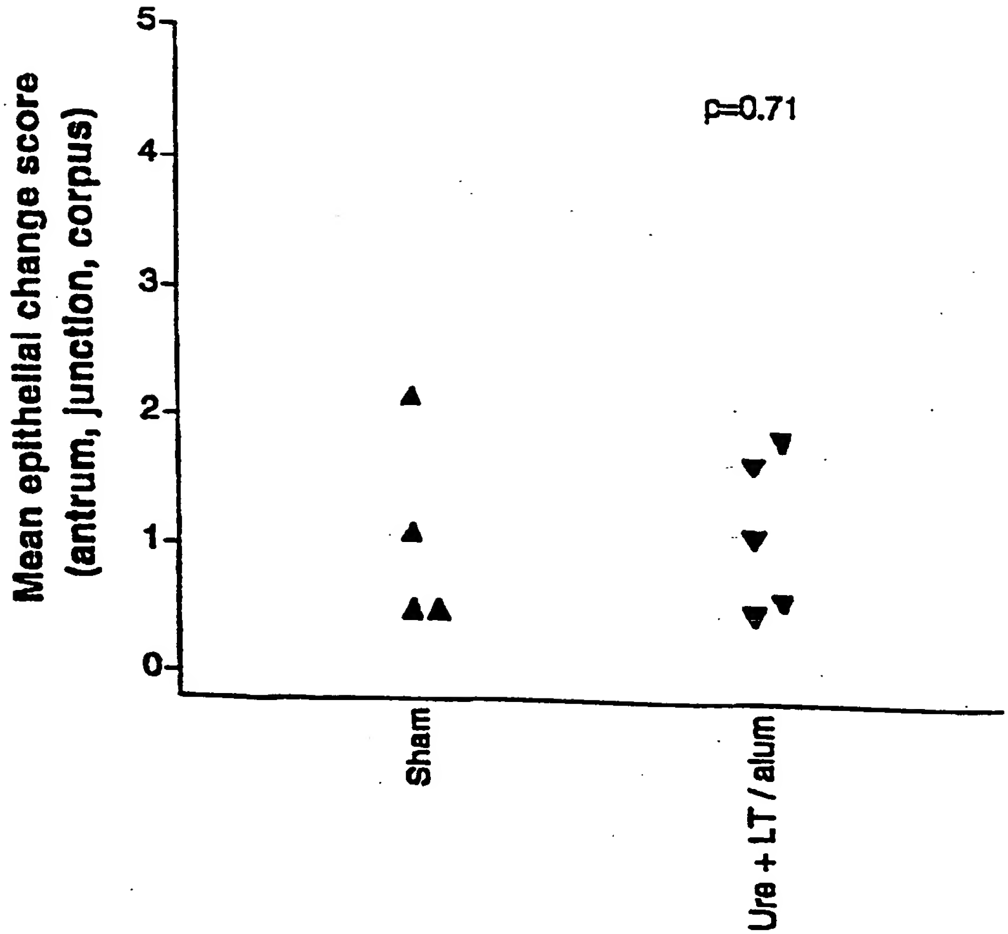

Mean gastritis score (antrum, junction, corpus)
5
4
3
2
1
0
p=0.32
Sham
Ure + LT / alum

Figure 9


Mean epithelial change score
(antrum, junction, corpus)
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4
3
2
1
0
p=0.71
Sham
Ure + LT / alum

Figure 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/08890

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 39/02; A01N 43/04; A61K 31/70
US CL : 514/44; 424/234.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 424/234.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, MEDLINE
search terms:helicobacter?, pylori or pyloridis or pyloris?, th1, th2, mucosal?, oral?, parenteral?, adjuvant?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 96/38475 A1 (ASTRA AKTIEBOLAG) 05 December 1996, see page 11, lines 17-26, pages 12-13, and claims 1-25, see entire document.	1-9, 25-36
Y	WO 95/33482 A1 (CSL LIMITED) 14 December 1995, see paragraph linking pages 4-5, and claims 1-25, see entire document.	1-9, 25-33
Y	KAPLAN, L. Autogenous vaccination against Helicobacter- pylori. South African Medical Journal. December 1993, Vol. 83, No. 12, pages 922-923, especially page 923, see entire document.	1-9, 25-33

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
03 JUNE 1998	06 AUG 1998

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	GINNY PORTNER Dorothea Lawrence For
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/08890

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 10-24
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 93/18150 A1 (BIOCINE SCLAVO S.P.A.) 16 September 1993, see page 41, lines 8-17, see entire document.	1-9, 25-33
Y	WO 96/40893 A1 (ASTRA AKTIEBOLAG) 19 December 1996, see page 83, lines 29-39, page 86, lines 1-29, page 103, lines 15-23, see entire document.	1-9, 25-33
Y	FERRERO, R.L. et al. Recombinant antigens prepared from the urease subunits of Helicobacter spp.:Evidence of protection in a mouse model of Gastric Infection. Infection and Immunity. November 1994, Vol. 62, No. 11, pages 4981-4989, see abstract and entire document.	1-9, 25-33
Y	LEE, C.K. et al. Oral immunization with recombinant Helicobacter pylori urease induces secretory IgA antibodies and protects mice from challenge with Helicobacter felis. The Journal of Infectious Diseases, July 1995. Vol. 172, pages 161-172, especially pages 165-169, see entire document.	1-9, 31-35
Y	US 5,610,060 A (WARD et al) 11 March 1997, see col. 4, lines 13-58, see entire document.	1-9, 25
Y	MOHAMMADI, M. et al. Helicobacter-specific cell-mediated immune responses display a predominant Th1 phenotype and promote a delayed-type hypersensitivity response in the stomachs of mice. Journal of Immunology, 15 June 1996, Vol. 156, No. 12, pages 4729-4738, see entire document.	1-9, 25-33
Y	D'ELIOS, M.M. et al. T helper 1 effector cells specific for Helicobacter pylori in the gastric antrum of patients with peptic ulcer disease. Journal of Immunology, 15 January 1997, Vol. 158, No. 2, pages 962-967, see entire document.	1-9, 25-33
Y	DOIG, P. et al. Identification of surface-exposed outer membrane antigens of Helicobacter pylori. Infection and Immunity. October 1994, Vol. 62, No. 10, pages 4526-4533, see page 4528, see entire document.	1-9
A,P	US 5,679,769 A (DANISHEFSKY et al) 21 October 1997, see col. 2, lines 26-40, col. 20, lines 46-64, see entire document.	1-9, 25-36

Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 11578–11583, September 1999
Microbiology

DNA adenine methylase mutants of *Salmonella typhimurium* show defects in protein secretion, cell invasion, and M cell cytotoxicity

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Communicated by Maurice S. Fox, Massachusetts Institute of Technology, Cambridge, MA, July 20, 1999 (received for review January 28, 1999)

ABSTRACT **Mutants of *Salmonella typhimurium* lacking DNA adenine methylase are attenuated for virulence in BALB/c mice. LD_{50} values of a DNA adenine methylation (Dam)$^-$ mutant are at least 10^3- to 10^4-fold higher than those of the parental strain when administrated by oral or intraperitoneal routes. Dam$^-$ mutants are unable to proliferate in target organs but persist in low numbers in these locations. Efficient protection to challenge with the virulent parental strain is observed in mice infected with a Dam$^-$ mutant. Use of the ileal loop assay shows that Dam$^-$ mutants are less cytotoxic to M cells and fail to invade enterocytes. In the tissue culture model, lack of DNA adenine methylation causes reduced ability to invade nonphagocytic cells. In contrast, no effect is observed either in intracellular proliferation within nonphagocytic cells or in survival within macrophages. The invasion defect of Dam$^-$ mutants is correlated with a distinct pattern of secreted proteins, which is observed in both PhoP$^+$ and PhoP$^-$ backgrounds. Altogether, our observations suggest a multifactorial role of Dam methylation in *Salmonella* virulence.**

In the bacterial cell, DNA adenine methylation (Dam) modulates a variety of processes such as DNA replication, chromosome segregation, mismatch repair, and transcription of certain genes (1–3). Given these multiple roles, it is not surprising that *dam* mutations are highly pleiotropic; however, lack of Dam methylation does not impair viability (1, 3, 4). The existence of genes whose transcription is regulated by Dam methylation has been known for two decades in *Escherichia coli* and its phages (1–3), and novel cases have recently been described in *Salmonella typhimurium* (4–6). Genes regulated by Dam methylation usually contain GATC sites either in their promoter or in upstream regulatory sequences. The methylation state of these GATC sites controls the interaction between RNA polymerase or regulatory proteins and their cognate DNA-binding sites (1, 7). A paradigmatic case of transcriptional regulation by Dam methylation is the *E. coli pap* operon, which directs the synthesis of pili required for adhesion to uroepithelial cells. In uropathogenic *E. coli*, expression of *pap* is subjected to phase variation (8, 9). The ON and OFF stages are dictated by binding of the regulatory protein Lrp and accessory proteins to two GATC sites upstream of the transcription initiation site (8, 10). Recent work has shown that expression of other *E. coli* operons encoding virulence-related fimbriae, such as *sfa*, *daa*, and *fae*, are also regulated by Lrp binding (11, 12). As in the *pap* operon, Lrp mediates transcriptional control over these fimbrial operons depending on the methylation status of critical GATC sites (11, 12).

Dam$^-$ mutants of *Salmonella typhimurium* were recently described (4). The same study showed that Dam methylation regulates the expression of a locus in the *S. typhimurium* virulence plasmid (4). This finding and the fact that Dam methylation modulates the expression of virulence-related fimbriae in *E. coli* (10) led us to examine whether Dam methylation could play a role in *S. typhimurium* virulence. Tests in the BALB/c murine model were unambiguous: absence of DNA adenine methylation produces severe attenuation of *S. typhimurium* virulence. A partial defect in the capacity of *S. typhimurium* to invade nonphagocytic cells is also observed. Furthermore, Dam$^-$ mutants are unable to invade enterocytes and to cause cytotoxicity on M cells of ileal Peyer's patches. Our conclusion that DNA adenine methylation plays a major role in *S. typhimurium* virulence is supported by the recent finding that Dam$^-$ mutants of *Salmonella* show altered expression of a number of *in vivo*-induced genes (6).

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Growth Media, and Growth Conditions. *S. typhimurium* SL1344 was used as the standard virulent strain (13). Strains SV1610 (SL1344 *dam-228*::MudJ), SV4036 (*mutL111*::Tn*10*), SV4056 (*phoP7953*::Tn*10*) and SV4089 (*dam-228*::MudJ *phoP7953*::Tn*10*), all derived from SL1344, are described in this study. The allele *mutL111*::Tn*10* was obtained from G. C. Walker, Massachusetts Institute of Technology, Cambridge, MA. The allele *phoP7953*::Tn*10* was provided by E. A. Groisman, Washington University, St. Louis, MO. Plasmid pTP166, obtained from M. G. Marinus, University of Massachusetts, Worcester, MA, is a ColE1 derivative harboring the wild-type *dam* gene of *E. coli* under the control of a *tac* promoter (14). Noninvasive *S. typhimurium* strains SB220 (SL1344 *sipC*::*aph*) and SB302 (SL1344 *invJ*::*aph*) were provided by J. E. Galán, Yale University, New Haven, CT (15, 16). Strains were grown overnight in LB broth at 37°C without shaking before administration to BALB/c mice, infection of tissue culture cells, or preparation of extracts containing secreted proteins (see below). Solid LB contained 1.5% Difco agar. Green plates were prepared as described elsewhere (4).

Virulence and Vaccination Assays in BALB/c Mice. Bacteria were centrifuged at 10,000 × *g* for 15 min and washed twice in sterile PBS. Serial dilutions were used to infect orally (25 μl) or intraperitoneally (200 μl) groups of 8-wk-old female BALB/c mice. In oral challenge experiments, the acidic pH of the stomach was buffered by suspending bacteria in a 2.5% bicarbonate–0.2% lactose solution before administration. Survival of infected mice was recorded for a minimum of 4 wk. LD_{50} was calculated by the method of Reed and Muench (17). In vaccination studies 4 wk after infection with the *S. typhimurium* Dam$^-$ mutant SV1610, mice were challenged orally

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Abbreviation: Dam, DNA adenine methylation.

‡To whom reprint requests should be addressed. E-mail: genbac@cica.es.

with different doses of the parental virulent strain SL1344. Mouse survival was recorded for 4 additional weeks. In organ colonization studies, lymph nodes, liver, and spleen were removed from two mice at each postinfection time and homogenized in 10 ml of sterile PBS. Dilutions of these homogenates were plated on LB to count the numbers of viable bacteria per organ.

Ligated Loop Model and Electron Microscopy. Interaction of wild-type and Dam⁻ bacteria with murine intestinal epithelium was examined as described by Jones *et al.* (18). Briefly, 7-wk-old BALB/c mice were starved for 24 h and anesthetized before surgery by i.p. injection of 2.0 mg of Nembutal. On practicing a small incision, the small bowel was exposed, and a loop was formed by ligating the intestine with silk thread at the ileocaecal junction and at a site 5 cm proximal to the caecum. Two hundred microliters of bacteria (*ca.* 4×10^8 colony-forming units) were then injected through a 25-gauge needle. The bowel was returned to the abdomen and the incision stapled. Mice were kept alive for 3 h and then sacrificed. The bacteria-exposed intestinal loop was removed, and sections containing Peyer's patches were fixed in a solution of 2.5% glutaraldehyde/0.8% paraformaldehyde in 0.1 M phosphate buffer. After 15 min, Peyer's patches were displayed in fresh fixing solution for an additional hour. Postfixation was made with 0.1% osmium tetroxide for 1 h. After three washes with phosphate buffer, samples were dehydrated with ethanol and propylene oxide. Infiltration and embedding was achieved with Epon resin. Resin polymerization was allowed for 48 h at 60°C. Ultrathin sections were contrasted with uranyl acetate and lead and examined in a JEOL1200EX electron microscope.

Eukaryotic Cell Lines. HeLa epithelial cells (ATCC CCL2) and J774.A1 mouse macrophages were used as prototypes of nonphagocytic and phagocytic cells, respectively. Mouse resident peritoneal macrophages were harvested from BALB/c mice as described by Leung and Finlay (19). Cells were grown in MEM containing 10% fetal bovine serum (HeLa epithelial cells) or in DMEM containing 5% fetal bovine serum and 1 mM glutamine (macrophages).

Bacterial Infection of Eukaryotic Cells. Eukaryotic cells were seeded the day before the infection in 24-well plates and grown at 37°C, 5% CO_2 to obtain 80% confluency. Bacteria were added to reach a multiplicity of infection of 10:1 bacteria/eukaryotic cell. Eukaryotic cells were infected for 15 min (HeLa epithelial cells), 20 min (resident peritoneal macrophages), or 10 min (J774.A1 macrophages) to obtain an infection rate of 70–80% of the cells present. Infected cells were washed three times with PBS and incubated in fresh tissue culture medium containing 100 μg/ml gentamicin. Two hours postinfection, the concentration of gentamicin was lowered to 10 μg/ml. Numbers of viable intracellular bacteria were obtained by lysing infected cells with 1% Triton X-100 and subsequent plating (20). Invasion rates of nonphagocytic cells were determined as the ratio of viable intracellular bacteria at a short postinfection time (2 h) vs. viable bacteria added to infect the eukaryotic cells.

Extracts of Secreted Bacterial Proteins. Extracts of secreted proteins were prepared by the method of Kaniga *et al.* (15). Proteins were analyzed by SDS/PAGE in Tris/Tricine buffer by using 8% acrylamide gels (21).

RESULTS

***S. typhimurium* Dam⁻ Mutants Are Attenuated for Virulence in the BALB/c Mouse Model.** To examine whether Dam methylation is required for virulence in the murine typhoid model, BALB/c mice were infected with various doses of a Dam⁻ strain of *S. typhimurium* (SV1610) by oral and i.p. routes. In parallel, infections were performed with the virulent strain SL1344 (13). Table 1 shows that LD_{50} values obtained for the Dam⁻ mutant were over 8.8×10^8 bacteria (oral) and 6.5×10^4 bacteria (i.p.). No orally challenged mice died or showed any symptom of disease during the course of the experiment (4 wk). The LD_{50} values obtained (Table 1) were at least 10^3- to 10^4-fold higher than those of the parental strain SL1344, in agreement with a recent study (6). To confirm that the attenuation of virulence observed in the Dam⁻ mutant was exclusively caused by lack of Dam methylation, we constructed a SV1610 derivative carrying plasmid pTP166, which contains the wild-type *dam* gene from *E. coli* (14). The complemented strain was fully virulent (Table 1). Complementation failed when the strain carried high-molecular-weight multimers of pTP166 (data not shown). The latter phenomenon was interpreted as an artifact and was not further investigated. However, the possibility that abnormally high levels of Dam methylase might also cause virulence defects, as suggested by Heithoff *et al.* (6), can be considered. Complementation of virulence in the *in vivo* model shows that there is no polar effect of the Mu*d*J element inserted in the *dam* gene on hypothetical virulence genes located downstream of *dam*. Altogether, these data show that *S. typhimurium* requires Dam methylation to cause disease in the murine model. Such a requirement is unrelated to the mismatch repair deficiency of Dam strains, because a MutL⁻ mutant (SV4036) proved to be virulent (Table 1).

Table 1. Virulence properties of a *S. typhimurium* Dam⁻ mutant in BALB/c mice

Bacterial strain	LD_{50} (oral)*	LD_{50} (i.p.)
SL1344 (wild type)	6×10^4	<10
SV1610 (Dam⁻)	$>8.8 \times 10^8$	6.5×10^4
SV1610/pTP166	2.75×10^5	<35
SV4036 (MutL⁻)	2.4×10^6	<17

*Oral inoculation was done by suspending bacteria in bicarbonate buffer before administration to mice.

Colonization of Target Organs of Infected Mice by the *S. typhimurium* Dam⁻ Mutant. Because there was no sign of disease after oral and i.p. challenge with strain SV1610 (Dam⁻), we reasoned that Dam methylation could be required for colonization of target organs. To test this possibility, mice were infected orally and intraperitoneally. Lymph nodes, liver, and spleen were harvested periodically from day 1 to 21 after infection, and viable bacteria were counted by plating. Low numbers of Dam⁻ bacteria, approximately 10^2–10^3, were found in these organs 3 wk after infection (Fig. 1). In contrast, the parental (virulent) strain SL1344 increased by a factor of 10^3–10^4 in number during the first week of infection (Fig. 1). On i.p. challenge, Dam⁻ bacteria increased in number during the first week, dropping afterward to a steady value of 10^2–10^3. The complemented strain SV1610/pTP166 (*dam-228*::Mu*d*J/*dam*⁺) was likewise able to grow within organs on oral administration (data not shown). Altogether, these data suggest that DNA adenine methylation is essential for *S. typhimurium* to proliferate within target organs on oral and i.p. administration.

A *S. typhimurium* Dam⁻ Mutant Elicits Protection to Challenge with the Virulent Strain. The fact that the Dam⁻ mutant is able to persist, albeit in low numbers, in target organs for 3 wk (Fig. 1) led us to examine whether this attenuated strain could elicit a specific immune response. Mice were infected orally or intraperitoneally with different doses of strain SV1610 (Dam⁻) and challenged 4 wk after infection with high doses of the virulent strain SL1344. Table 2 shows that infection of BALB/c mice with the Dam⁻ mutant rendered the animal capable of surviving to lethal doses of the virulent strain. Of a total of 42 mice infected with the Dam⁻ mutant, 38 survived the challenge with an amount of virulent bacteria up to 100-fold the LD_{50} (Table 2). Interestingly, as few as 89 Dam⁻ *S. typhimurium* cells administered by the i.p. route were

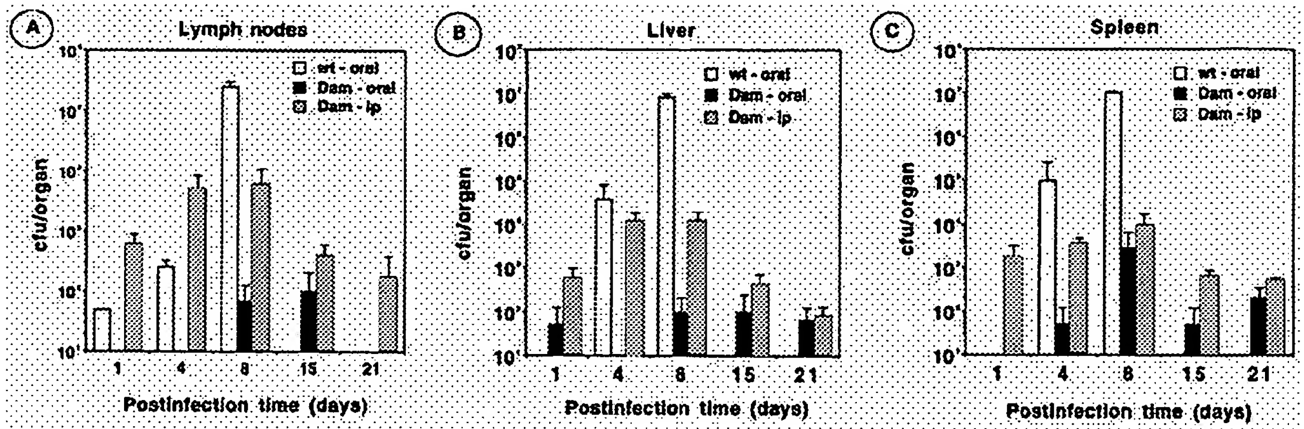

FIG. 1. Colonization of mouse organs by a *S. typhimurium* Dam$^-$ mutant. Groups of 8-wk-old BALB/c mice were infected orally with 8.8×10^8 colony-forming units (cfu) of the strain SV1610 (Dam$^-$) [black histograms] or 7.5×10^5 cfu of strain SL1344 (wild type) [white histograms]. Another group of mice were infected intraperitoneally with 1.5×10^5 cfu of SV1610 (Dam$^-$) [grey histograms]. At the times indicated, lymph nodes (*A*), liver (*B*), and spleen (*C*) were removed from two infected mice, and viable bacteria were determined by plating. At 8 days after infection, all the wild-type infected mice showed clear evidence of disease.

capable of eliciting in the animal a specific immune response against the virulent strain (Table 2).

Behavior of the *S. typhimurium* Dam$^-$ Mutant in the Tissue Culture Model. Dam$^-$ mutants of *S. typhimurium* do not exhibit growth defects in standard media (4). In addition, the Dam$^-$ strain used in this study, SV1610, shows a normal serum-resistance response (F.G-P., unpublished work). Therefore, we reasoned that attenuation of the Dam$^-$ mutant might reflect an impaired interaction of bacteria with host cells. To test this hypothesis, we analyzed whether the Dam$^-$ mutant showed defects in invasion, survival, or growth within nonphagocytic and phagocytic cells. Table 3 shows that the Dam$^-$ mutant is partially impaired for invading nonphagocytic cells (30% of wild-type values in HeLa cells). The complemented strain showed wild-type invasion ability (Table 3). Well known invasion mutants such as SipC$^-$ and InvJ$^-$ yielded values less than 1% (Table 3). The Dam$^-$ mutant did not show any proliferation defect within HeLa cells (Table 3). To test whether Dam methylation was required for bacterial survival within macrophages, both the mouse macrophage cell line J774A.1 and mouse resident peritoneal macrophages were used. As previously reported, strain SL1344 was able to proliferate within J774A.1 and to survive within peritoneal macrophages (ref. 22; see also Table 3). Neither the Dam$^-$ nor the Dam$^-$/Dam$^+$ (complemented) strain showed any noticeable defect in the interaction with macrophages (Table 3). These data suggest that DNA adenine methylation is not essential for bacteria to proliferate within nonphagocytic cells or to survive within macrophages. In contrast, Dam methylation seems to be required to trigger an efficient bacterial uptake by nonphagocytic cells.

Table 2. Protection conferred by a *S. typhimurium* Dam$^-$ mutant to challenge with the virulent strain

Experiment	Dose of SV1610 (Dam$^-$)*	Challenge dose of SL1344 (wt)†	No. dead mice/ inoculated mice
1	—	2.8×10^3	1/5
	—	2.8×10^4	1/5
	—	2.8×10^5	4/5
2	2.5×10^6	1.2×10^6	1/4
	2.5×10^7	1.2×10^6	1/4
	2.5×10^8	1.2×10^6	0/4
3	7×10^6	9.7×10^6	0/5
	7×10^7	9.7×10^6	1/5
	7×10^8	9.7×10^6	0/5
	89 (i.p.)	9.7×10^6	0/5
	890 (i.p.)	9.7×10^6	0/5
	8,900 (i.p.)	9.7×10^6	1/5

*Inoculations were done by the oral route, except when indicated i.p. After 4 wk, all Dam$^-$ infected mice survived the doses of bacteria indicated.

†All inoculations were done by the oral route. In experiments 2 and 3, challenges with the wild-type strain were done 4 wk after the inoculation with the Dam$^-$ mutant. Mouse survival was recorded for 4 additional weeks.

Phenotype of a Dam$^-$ Mutant in the Murine Intestinal Epithelium. Because Dam$^-$ mutants showed a partial defect in invasion of cultured epithelial cells, we addressed the murine ligated loop assay to monitor two critical events linked to *Salmonella* penetration of the intestinal epithelium: cytotoxicity on M cells of Peyer's patches and invasion of enterocytes. Elicitation of both processes has been shown to be related to the invasion capacity observed in the *in vitro* models (23–25). Disruption of M cell integrity was observed on 3 h of infection with the wild type (Fig. 2 *a* and *b*). In contrast, very few Dam$^-$ (SV1610) bacteria were seen interacting with M cells and, in the rare cases observed, the presence of bacteria did not trigger M cell cytotoxicity (Fig. 2*c*). Moreover, unlike the wild type, Dam$^-$ cells were not seen either causing massive destruction of the intestinal epithelium or moving to deep locations close to the *lamina propria* (Fig. 2*d*). Another distinct phenotype of the Dam$^-$ mutant was its impaired capacity to invade enterocytes (Fig. 2 *e* and *f*). Altogether, these data suggest that Dam methylation modulates the interaction of *Salmonella* with different cell types in Peyer's patches.

Secretion of Proteins in *S. typhimurium* Dam$^-$ Mutants. The virulence trait affected in Dam$^-$ mutants, invasion of nonphagocytic cells, has been shown to depend on a specialized type III secretion system (reviewed in refs. 26, 27). Type III-secreted proteins have been identified in bacterial culture media supernatants and shown essential for *Salmonella* invasion (26, 27). We thus analyzed the protein content of supernatants of the wild type, a Dam$^-$ mutant, and a Dam$^+$-complemented strain. Extracts containing *Salmonella*-secreted proteins (ssp) were examined by Coomassie staining. The protein profile obtained for the Dam$^-$ mutant was clearly different, and at least three proteins found in the Dam$^-$ mutant were not detectable in the wild type (Fig. 3). Interestingly, the *dam* mutation causes a reduction in the secretion of SPI-1 encoded effector proteins, such as SipA, SipB, and SipC, whereas flagellin secretion is unaffected (Fig. 3). Complementation of the *dam* mutation fully restored the profile of ssp observed in the wild type (Fig. 3).

Table 3. Phenotype of a Dam⁻ mutant in the tissue culture model

Strain	Invasion, %†	Intracellular proliferation‡	Survival in macrophages*	
			J774.A1	Mouse peritoneal
SL1344 (wt)	100 ± 23.8	25.3 ± 5.05	6.85 ± 0.3	0.41 ± 0.13
SV1610 (Dam^-)	28.4 ± 9.2	16.1 ± 3.4	7.7 ± 1.65	0.22 ± 0.12
SV1610/pTP166 (Dam^-/Dam^+)	105.4 ± 33.9	28.9 ± 0.3	10 ± 0.53	0.33 ± 0.05
SB220 ($SipC^-$)	0.43 ± 0.05	ND	ND	ND
SB302 ($InvJ^-$)	0.65 ± 0.23	ND	ND	ND

*Ratio of viable intracellular bacteria at 20 h vs. 0.5 h after infection. In both types of macrophages, cells were infected for 10 min.

All values are averages of triplicate samples from at least two independent trials. ND, not determined.

†Percentage of bacteria that invade HeLa epithelial cells during 15 min and survived a gentamicin protection assay. Plating was done at 2 h after infection. Values were normalized to invasion rate of wild-type bacteria (0.109 ± 0.02).

‡Ratio of viable intracellular bacteria at 24 h vs. 2 h after infection, obtained after incubating HeLa cells with bacteria for 15 min.

Because an interconnection of regulons under Dam and PhoP control has been recently postulated (6), we examined the effect of *dam* and *phoP* mutations on secreted protein profiles. In agreement with previous observations (28), a $PhoP^-$ mutant showed a wild-type profile of ssp. In contrast, the ssp profile of a Dam^- $PhoP^-$ double mutant was similar to that of a Dam^- mutant (Fig. 3). Thus, the secretion defect of Dam^- mutants is observed in both the presence and the absence of the PhoP product. The observation that the role of Dam methylation in secretion is PhoP independent can be tentatively correlated with invasion assays in HeLa cells: a *dam* mutation caused a similar invasion reduction in both $PhoP^+$ and $PhoP^-$ backgrounds (data not shown). Furthermore, we investigated the involvement of Dam methylase on secretion of SPI1-encoded proteins essential for invasion, such as SipC and InvJ. Western analysis of ssp supernatant extracts by using specific anti-SipC and -InvJ antibodies indicated that secretion of both proteins is reduced by 50% in the Dam^- mutant (data not shown). Globally, these assays suggest that Dam methylation might modulate the SPI-1 encoded type III secretion system.

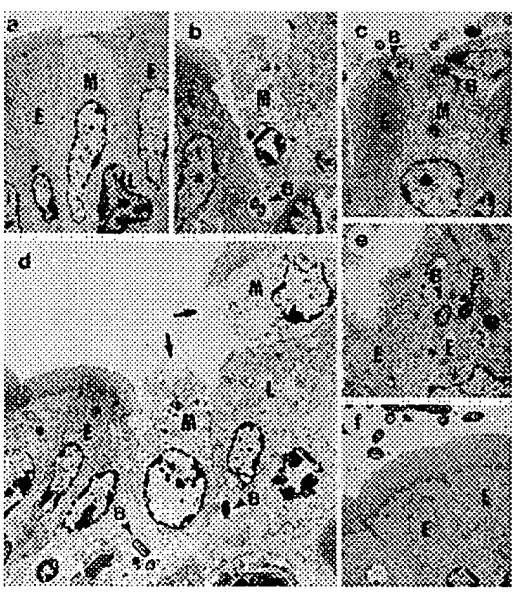

FIG. 2. Interaction of *S. typhimurium* Dam^- mutants with the murine intestinal epithelium. The ligated loop assay was used to examine the interaction of SL1344 (wild type) and SV1610 (Dam^-) with cell types present in Peyer's patches 3 h after infection. Shown are transmission electron micrographs of: (*a*) an uninfected M cell; (*b*) an M cell disrupted by the infection of the wild-type strain; (*c*) Dam^- bacteria interacting with M cells; note the lack of cytotoxicity on the M cell; (*d*) massive destruction of the intestinal epithelium, which now appears with a clear gap (shown by arrows) as a result of infection by wild-type bacteria. This generalized destruction was never observed in loops infected with Dam^- bacteria; (*e*) wild-type bacteria internalized by enterocytes; (*f*) Dam^- bacteria that do not invade enterocytes. M, M cell; E, enterocyte; L, lymphocyte; B, intracellular bacteria. Note the presence of bacteria in deep locations only in tissues infected with the wild-type strain (*b* and *d*). (*a–d*, ×2,000; *e* and *f*, ×3,000.)

DISCUSSION

In the murine model, attenuation of *S. typhimurium* virulence in the absence of Dam methylation is observed in both the oral and the intraperitoneal routes. *In vivo* studies have also shown that, on oral and i.p. administration, the Dam^- mutant is able to colonize target organs, but not to proliferate in these locations. The combination of attenuated virulence and organ colonization ability makes Dam^- mutants excellent candidates for vaccination. In fact, the Dam^- mutant used in this study was able to elicit an immune response sufficient to prevent killing by the virulent strain at doses 100-fold the LD_{50}. Similar observations have recently been reported by Heithoff *et al.* (6).

Given the high pleiotropy of *dam* mutations (1, 4), the specific link(s) between Dam methylation and systemic disease cannot be established *a priori*. However, insights were obtained from the ileal ligated loop assay: the Dam^- mutant does not cause cytotoxicity on M cells and is unable to invade enterocytes. These observations explain the low numbers of bacteria recovered from target organs on oral challenge. The M cell has been shown to play a critical role in the intestinal immune response (29). Therefore, lack of cytotoxicity on M cells, together with their proper stimulation by infecting Dam^- bacteria, can improve the host immune response and render the Dam^- mutant avirulent. In fact, it is known that noninvasive *S. typhimurium* mutants are avirulent because they do not destroy M cells (30). These results can also explain the optimal vaccine properties of Dam^- mutants observed by us and by others (6).

Further insights on the role of Dam methylation in *Salmonella* virulence were obtained from the tissue culture model: a Dam^- mutant showed reduced capacity to invade HeLa cells. However, the invasion impairment of a Dam^- mutant was only partial, compared with a SPI1 secretion-deficient $InvJ^-$ mutant or a mutant lacking the effector invasion protein SipC. These differences may indicate that a *dam* mutation causes mild changes in the amount or the activity of these proteins, compared with the effect of knocking out the corresponding genes.

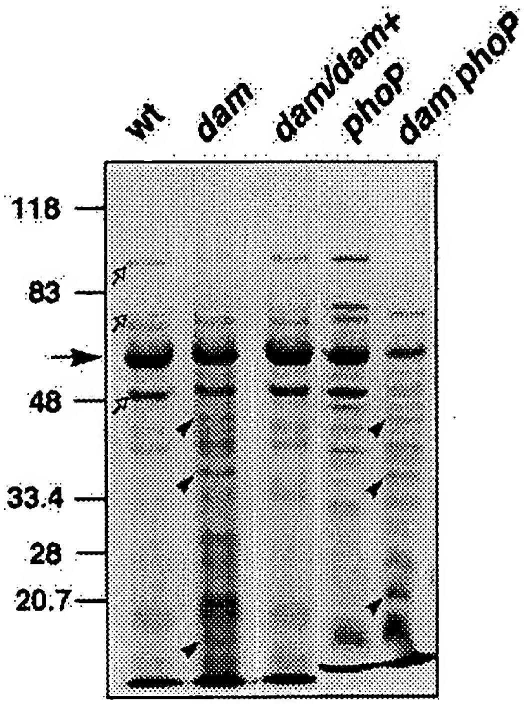

FIG. 3. Lack of Dam methylation alters protein secretion. An SDS gel with 8% tricine showing proteins present in culture supernatants from SL1344 (wild type), SV1610 (Dam$^-$), SV1610/pTP166 (Dam$^-$/Dam$^+$), SV4056 (PhoP$^-$), and SV4089 (Dam$^-$ PhoP$^-$) strains. Indicated are flagellin (filled arrow), SipA, SipB, and SipC proteins (empty arrows), and three proteins present in supernatants of Dam$^-$ strains (arrowheads).

Because secreted proteins are well known effectors in the interaction with eukaryotic cells (26), we considered the possibility that absence of Dam methylation might cause changes in protein secretion mediated by the SPI1 type III system. To test this hypothesis, protein profiles were analyzed in culture supernatants. A distinct pattern of protein secretion was observed in the Dam$^-$ mutant. The most relevant differences were: (*i*) the presence of proteins absent from wild-type extracts and (*ii*) a reduction in the level of secreted SipA, SipB, and SipC. No defect was observed in flagellin secretion, implying that the effects observed are specific for secretion via the virulence-related type III apparatus. To our knowledge, this is the first description of a DNA modification mechanism that influences secretion of virulence determinants. The effect of Dam methylation on the SPI1 secretion system could be exerted on structural or regulatory genes. Putative target SPI-1-encoded regulators include HilA, InvF (31, 32), and the recently described HilC and HilD (33). Alternatively, Dam methylation could be essential for the activity of other regulators acting on (but not encoded by) SPI1, such as SirA (34, 35) or PhoPQ (28).

Although our protein analyses imply a connection between Dam methylation and the functionality of the SPI1 type III secretion system, our virulence data clearly indicate that the Dam$^-$ mutant is also attenuated when administrated intraperitoneally. Because SPI1-defective mutants are still virulent when tested by this route (30, 36, 37), other major virulence genes involved in *Salmonella* systemic disease might be controlled by Dam methylation. Putative candidates are components of the second type III system encoded in SPI2 (38, 39) or alternative sigma factors as RpoS (σ^S) or RpoE (σ^E) (40, 41). However, unlike mutants in these components or regulators, which show clear defects in intracellular proliferation (38, 39) or intracellular survival within macrophages (38–41), our study shows that Dam$^-$ mutants are not deficient in these processes. Therefore, further characterization of genes modulated by Dam methylation is required to ascertain which gene products essential for systemic infection are affected in Dam$^-$ mutants. A recent report by Heithoff *et al.* (6) proposes that a large number of Dam-regulated virulence genes exist in *S. typhimurium*, thus supporting our view that the effects of DNA adenine methylase mutations on *Salmonella* virulence are pleiotropic.

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Title: An Essential Role for DNA Adenine Methylation in Bacterial Virulence.

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An Essential Role for DNA Adenine Methylation in Bacterial Virulence

Salmonella typhimurium lacking DNA adenine methylase (Dam) were fully proficient in colonization of mucosal sites but showed severe defects in colonization of deeper tissue sites. These Dam^- mutants were totally avirulent and were effective as live vaccines against murine typhoid fever. Dam regulated the expression of at least 20 genes known to be induced during infection; a subset of these genes are among those activated by the PhoP global virulence regulator. PhoP, in turn, affected Dam methylation at specific genomic sites, as evidenced by alterations in DNA methylation patterns. Dam inhibitors are likely to have broad antimicrobial action, and Dam^- derivatives of these pathogens may serve as live attenuated vaccines.

Methylation at adenine residues by Dam controls the timing and targeting of important biological processes such as DNA replication, methyl-directed mismatch repair, and transposition (1). In addition, Dam regulates the expression of operons such as pyelonephritis-associated pili (pap), which are an important virulence determinant in upper urinary tract infections (2,3). The latter regulatory mechanism involves formation of heritable DNA methylation patterns, which control gene expression by modulating the binding of regulatory proteins. Although Dam regulates pili gene expression, its role in microbial pathogenesis has never been tested.

To determine whether Dam plays a role in the pathogenesis of Salmonella typhimurium, we assessed the effect of an insertion in the dam gene (Mud-Cm). The oral lethal dose required to kill 50% of the animals (LD_{50}) of this Dam^- mutant was more than 10,000 times the LD_{50} for the wild type, and the intraperitoneal LD_{50} for the mutant was more than 1000 times that for the wild type (Table 1). Because the dam insertion could decrease the expression of downstream genes (polar effects), an in-frame, nonpolar dam deletion was constructed (4) and shown to have the same reduced virulence as the dam insertion. Thus, the attenuation

was specifically attributable to the lack of Dam. Moreover, intraperitoneal inoculation of mice with a mixture of equal numbers of Dam^+ and Dam^- Salmonella showed that Dam^- mutants were completely eliminated during growth in the mouse (competitive index assay). Similar results were obtained with a strain that overproduces Dam from a recombinant plasmid, which suggested that precise amounts of the Dam methylase are required for full virulence. These results show that the Dam methylase is essential for bacterial pathogenesis.

Dam plays an essential role in methyl-directed mismatch repair (MDMR) because it allows discrimination between parental and daughter DNA strands (1). Thus, in the absence of Dam, bacteria show an increased mutation rate. To test the hypothesis that the reduction in virulence of Dam^- Salmonella was due to a high mutation rate, we measured the virulence of mutS Salmonella, which lack MDMR and also have a high mutation rate. Table 1 shows that in both the oral LD_{50} and the competitive index virulence assays, mutS Salmonella were identical to the wild type, indicating that Dam does not affect pathogenesis via an increased mutation rate. Because more DNA exchange between species occurs in $MutS^-$ strains than in $MutS^+$ strains, they more readily acquire new virulence determinants (1). The fact that $MutS^-$ strains are fully virulent could explain the high frequency at which mutS Escherichia coli and Salmonella mutants are found among clinical isolates (5).

Dam controls the expression of Pap pili by modulating the binding of leucine-responsive regulatory protein (Lrp) to pap regulatory DNA sequences (3). Lrp is a global regulator of at least 35 genes in E. coli that include operons involved in metabolism, transport, and adhesion (6). To determine whether Dam affects Salmonella virulence through an Lrp-mediated pathway, we analyzed Lrp^- Salmonella (Table 1). Salmonella lacking Lrp were fully virulent, as assessed by the LD_{50} and competitive index assays. These data show that Lrp is not required for virulence in a mouse model of typhoid fever.

The results discussed above show that adenine methylation is critical for Salmonella pathogenesis. DNA methylation of cytosine residues appears to be important for the regulation of biological processes in both plants and animals. Although Salmonella contain a DNA cytosine methylase (Dcm), the role of cytosine methylation in this organism is unclear. The dcm^- mutant was virulent in the LD_{50} and competitive index assays (Table 1). These results demonstrate that methylation of adenine but not cytosine residues is required for Salmonella pathogenesis.

DNA adenine methylation has been shown to directly control virulence gene expression (7). Therefore, we determined whether Dam regulates Salmonella genes that are preferentially expressed in the mouse [designated as in vivo-induced (ivi) genes (8-11)]. Dam significantly repressed the expression of more than 20 ivi genes (by a factor of 2 to 18) when grown in rich medium (Fig. 1). Four of the eight fusions in Fig. 1 are in known genes, all of which have been shown to be involved or have been implicated in virulence: spvB resides on the Salmonella virulence plasmid and functions to facilitate growth at systemic sites of infection (12); pmrB is involved in resistance to antibacterial peptides termed defensins (13); and mgtA and entF are involved in the transport of magnesium and iron, respectively (14,15). Additional ivi genes of unknown function were also Dam-regulated. These results indicate that Dam is a global regulator of Salmonella gene expression and that the dam-regulated ivi genes constitute a dam regulon (1).

Salmonella pathogenesis is known to be controlled by PhoP, a DNA binding protein that acts as both an inducer and repressor of specific virulence genes [reviewed in (16)]. To determine whether the Dam and PhoP regulatory pathways share common genes, we tested the effect of Dam on seven PhoP- activated ivi genes, including spvB, pmrB, and mgtA. Dam repressed the expression of these three genes by a factor of 2 to 19 (Fig. 2), and this repression was not dependent on the PhoP protein. Dam did not significantly affect the expression of the remaining four PhoP-activated genes (17). These results indicate that Dam and PhoP constitute an overlapping global regulatory network controlling Salmonella virulence.

Binding of regulatory proteins to DNA can form DNA methylation patterns by blocking the methylation of specific Dam target sites (GATC sequences) (18). Therefore, we further investigated the interactions between Dam and PhoP by determining whether the binding of PhoP (or a PhoP-regulated protein) to specific DNA sites blocks methylation of these sites by Dam, resulting in an alteration in the DNA methylation pattern. Analysis of $PhoP^+$ and $PhoP^-$ Salmonella showed distinct differences in DNA

methylation patterns. Digestion of genomic DNA from PhoP⁻ bacteria with Mbo I (which cleaves only at nonmethylated GATC sites) resulted in the appearance of DNA fragments that were not present in DNA from PhoP⁺ bacteria, indicating that the PhoP protein (or a PhoP-regulated gene product) blocks Dam methylation at specific GATC-containing sites in the Salmonella genome (Fig. 3, arrows). Recent data have shown that although catabolite gene activator protein binds to a DNA sequence containing GATC, it does not protect this site from methylation (18). Thus, not every protein that binds to a Dam target site protects the GATC sequence from methylation. It is also possible that PhoP⁺ and PhoP⁻ strains have different amounts of Dam activity, which in turn could affect DNA methylation patterns. However, this regulation does not occur at the transcriptional level because Dam does not alter PhoP expression, nor does PhoP alter Dam expression (17). Further analysis will determine whether these PhoP-protected sites are within regulatory regions of virulence genes, and whether DNA methylation directly affects the PhoP regulon by altering DNA-PhoP interactions.

In E. coli, almost all GATC sites protected from methylation are in 5' noncoding DNA regions presumably involved in the control of gene expression (19,20). Thus, it is likely that the DNA methylation patterns identified in Salmonella (Fig. 3) are also within gene regulatory regions. Methylation of specific GATC sites in the regulatory regions of virulence genes could affect the binding of regulatory proteins to DNA. Such altered protein-DNA interactions can affect gene expression, as has been shown for the pap virulence operon in E. coli (7,18). Similarly, Dam methylation could directly or indirectly affect the expression of PhoPQ-regulated genes in S. typhimurium.

Because Dam⁻ mutants were highly attenuated, we determined whether Dam⁻ Salmonella could serve as a live attenuated vaccine. Table 2 shows that all (17/17) mice immunized with a S. typhimurium Dam⁻ insertion strain survived a wild-type challenge of 104 above the LD50, whereas all nonimmunized mice (12/12) died after challenge. Moreover, because all (8/8) mice immunized with Salmonella containing the dam deletion survived challenge, these data indicate that protection was specifically due to the absence of Dam methylase. Preliminary experiments indicate that mice immunized with Dam⁻ S. typhimurium showed cross-protection against another pathogenic strain of Salmonella (17). The virulence attenuation and effectiveness of Dam⁻ mutants as a vaccine (Tables 1 and 2) could be due to the ectopic expression of virulence determinants (Figs. 1 and 2), which would likely be deleterious to the growth (or survival) of Salmonella during infection.

Dam⁻ Salmonella could have been avirulent as a result of multiple defects in basic cellular processes that reduced viability. This hypothesis was tested by comparing the survival of Dam⁺ and Dam⁻ Salmonella in mouse tissues. As shown in Fig. 4, Dam⁻ bacteria were fully proficient in colonization of a mucosal site (Peyer's patches) but showed severe defects in colonization of deeper tissue sites. Five days after infection, we observed a reduction of three orders of magnitude in numbers of Dam⁻ Salmonella in the mesenteric lymph nodes (relative to numbers of Dam⁺ bacteria) and a reduction of eight orders of magnitude in numbers of Dam⁻ Salmonella in the liver and spleen. These data show that Dam⁻ Salmonella survive in Peyer's patches of the mouse small intestine for at least 5 days, providing an opportunity for elicitation of a host immune response. Dam⁻ Salmonella, however, were unable to cause disease; they either were unable to invade systemic tissues or were able to invade but could not survive.

DNA adenine methylases are potentially excellent targets for both vaccines and antimicrobials. They are highly conserved in many pathogenic bacteria that cause significant morbidity and mortality, such as Vibrio cholerae (21), Salmonella typhi (22), pathogenic E. coli (23), Yersinia pestis (22), Haemophilus influenzae (24), and Treponema pallidum (25). In addition, because Dam is a global regulator of genes expressed during infection (Fig. 1), Dam⁻ mutants may ectopically express multiple immunogens that are processed and presented to the immune system. Such ectopic expression could elicit a cross-protective immune response between related bacterial strains that share common epitopes. Finally, because the Dam methylase is essential for bacterial virulence, Dam inhibitors are likely to have broad antimicrobial action, hence Dam is a promising target for antimicrobial drug development.

Table 1. Dam is required for Salmonella virulence. ND, not determined.

Legend for Chart:

A - Relevant genotype(*)
B - Oral LD_{50}(A)
C - Intraperitoneal LD_{50}(A)
D - Competitive index(B)

A	B	C	D
Wild type	10^5	<10	-
dam102::Mud-Cm	$>10^9$	$>10^4$	$<10^{-4}$
damDelta232 (nonpolar deletion)	$>10^9$	$>10^4$	$<10^{-4}$
Wild type (pTP166) (Dam overproducer)	10^8	$>10^4$	$<10^{-4}$
mutS121::Tn10	10^5	ND	0.9
lrp31::Km	10^5	ND	9.4
dcm1::Km	10^5	<10	0.2

(*) All bacterial strains used in this study are derivatives of S. typhimurium 14028. Mutant strains are isogenic to the wild type and were obtained or constructed as described (4). Strains used in infection studies were grown overnight in LB with shaking (9). The Dam-overproducing strain contains E. coli dam on a recombinant plasmid (pTP166) in a wild-type background (30).
(A) The LD_{50} assay for each of these strains was compared to that for the wild type. The peroral LD_{50} via gastrointubation for all derivatives was determined by infecting at least 12 BALB/c mice; the intraperitoneal LD_{50} was determined by infecting at least six mice. (B) At least five BALB/c mice were intraperitoneally infected with a 1:1 ratio of mutant to wild type, as described (9). Five days after infection, the bacterial cells were recovered from the spleen; the competitive index is the ratio of mutant to wild-type bacteria recovered.

Table 2. Dam- Salmonella serve as effective live attenuated vaccines.

Legend for Chart:

A - Immunization with Dam^- S. typhimurium(*)
B - Challenge with 10^9 wild-type S. typhimurium

A	B
None	12/12 dead
dam102::Mud-Cm	17/17 alive
damDelta232 (nonpolar deletion)	8/8 alive

(*) BALB/c mice were perorally immunized via gastrointubation with a dose of 10^9 Dam^- S. typhimurium. Five weeks later, the immunized mice were challenged perorally with 10^9 wild-type S. typhimurium as described. No visible effects of typhoid fever were observed after immunization with Dam^- Salmonella, nor were there visible effects after the wild-type challenge.

GRAPH: Fig. 1. Dam regulates in vivo induced genes. Beta-galactosidase expression from S. typhimurium ivi fusions in Dam^+ and Dam^- strains, grown to saturation in LB medium as described (27), was measured. The vertical axis shows Beta-galactosidase activities [micromoles of onitrophenol (ONP) formed per minute per A600 unit per milliliter of cell suspension x 10^3]. The Beta-galactosidase activities were assayed as described (28).

GRAPH: Fig. 2. Dam represses PhoP-activated genes. Beta-galactosidase expression from S. typhimurium ivi fusion strains, grown in minimal medium (pH 5.5, 50 MuM $Mg2^+$) as described (27), was measured. The vertical axis shows - galactosidase activities (calculated as in Fig. 1). The Beta-galactosidase activities were assayed as described (28).

GRAPH: Fig. 3. PhoP affects the formation of Salmonella DNA methylation patterns. DNA methylation patterns formed in PhoP+ and from PhoP- strains in minimal medium. Genomic DNA prepared from PhoP+ and from PhoP- strains embedded in agarose was cleaved with Mbo I (which cleaves nonmethylated Dam-target sites) and subjected to pulsed-field gel electrophoresis (29). The arrows indicate two DNA fragments that were present in $PhoP^-$ Salmonella but were absent in $PhoP^+$ Salmonella.

GRAPHS: Fig. 4. Colonization of mouse tissue sites by Dam^- Salmonella. BALB/c mice were infected via gastrointubation at a dose of 10^9 Dam^+ (open boxes) or Dam^- (closed boxes) S. typhimurium. After 1 day or 5 days after infection, mice were killed and bacteria were recovered from the host tissues indicated. PP, Peyer's patches (the four patches proximal to the ileal-cecal junction); MLN, mesenteric lymph nodes; CFU, colony-forming units.

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DNA Adenine Methylase Mutants of *Salmonella typhimurium* and a Novel Dam-Regulated Locus

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ABSTRACT

Mutants of *Salmonella typhimurium* lacking DNA adenine methylase were isolated; they include insertion and deletion alleles. The *dam* locus maps at 75 min between *cysG* and *aroB*, similar to the *Escherichia coli dam* gene. Dam⁻ mutants of *S. typhimurium* resemble those of *E. coli* in the following phenotypes: (1) increased spontaneous mutations, (2) moderate SOS induction, (3) enhancement of duplication segregation, (4) inviability of *dam recA* and *dam recB* mutants, and (5) suppression of the inviability of the *dam recA* and *dam recB* combinations by mutations that eliminate mismatch repair. However, differences between *S. typhimurium* and *E. coli dam* mutants are also found: (1) *S. typhimurium dam* mutants do not show increased UV sensitivity, suggesting that methyl-directed mismatch repair does not participate in the repair of UV-induced DNA damage in Salmonella. (2) *S. typhimurium dam recJ* mutants are viable, suggesting that the Salmonella RecJ function does not participate in the repair of DNA strand breaks formed in the absence of Dam methylation. We also describe a genetic screen for detecting novel genes regulated by Dam methylation and a locus repressed by Dam methylation in the *S. typhimurium* virulence (or "cryptic") plasmid.

THE biological functions of DNA adenine methylation have been widely investigated in *Escherichia coli* and its phages (reviewed by HATTMAN 1981; MARINUS 1984, 1987a,b; MESSER and NOYER-WEIDNER 1988; BARRAS and MARINUS 1989; NOYER-WEIDNER and TRAUTNER 1993). The DNA of *E. coli* contains ~1.5 mol of 6-methyl-adenine per 100 mol of adenine (HATTMAN 1981; MARINUS 1984). Formation of 6-methyl-adenine results from postreplicative modification of adenine residues in 5′-GATC-3′ sites; methylation occurs in both strands of the palindromic target (LACKS and GREENBERG 1977). The methylation reaction is catalyzed by the enzyme DNA adenine methyltransferase, often called "Dam methylase" (HERMAN and MODRICH 1982). The *dam* gene is located at 74 min on the *E. coli* genetic map and is cotransducible with *cysG* (MARINUS 1973; BACHMANN 1990). The *dam* gene is part of an operon containing *aroK, aroB, trpS* and two additional genes involved in carbohydrate metabolism (LØBNER-OLESEN *et al.* 1992; LYNGSTADAAS *et al.* 1995).

In *E. coli* and its phages Mu and P1, many roles of Dam methylation have been identified. Upon chromosome replication, the existence of hemimethylated 5′-GATC-3′ sites directs mismatch repair toward the newly synthesized, unmethylated strand (LU *et al.* 1983; PUKKILA *et al.* 1983; RADMAN and WAGNER 1986; MODRICH 1987). Dam methylation also controls the initiation of chromosome replication (MESSER *et al.* 1985; SMITH *et al.* 1985; BOYE and LØBNER-OLESEN 1990; CAMPBELL and KLECKNER 1990; ABELES *et al.* 1993), segregation of the daughter chromosome molecules (OGDEN *et al.* 1988; HERRICK *et al.* 1994), regulation of plasmid replication (RUSSEL and ZINDER 1987; GAMMIE and CROSA 1991), and the activity of certain host and phage genes (HATTMAN 1982; MARINUS 1985; ROBERTS *et al.* 1985; BRAUN and WRIGHT 1986; KÜCHERER *et al.* 1986; BLYN *et al.* 1990; CAMPBELL and KLECKNER 1990; BRAATEN *et al.* 1994). In addition, Dam methylation is involved in the initiation of P1 DNA replication (ABELES *et al.* 1993), packaging of bacteriophage P1 DNA into virions (STERNBERG and COULBY 1990) and also affects functions associated with bacterial retroposons (HSU *et al.* 1990). This variety of processes affected by Dam methylation confirms earlier expectations on its relevance as a physiological signal (HATTMAN 1981; MARINUS 1987a,b).

A *Salmonella typhimurium* point mutant lacking DNA adenine methylase was described a decade ago (RICHTIE *et al.* 1986). Given the pleiotropy of *dam* mutations of *E. coli* and the intricacies found in their manipulation (MCGRAW and MARINUS 1980; PETERSON *et al.* 1985; PETERSON and MOUNT 1993), we judged that the study of Dam methylation in Salmonella might become easier if insertion and deletion alleles were available. Both types of alleles are described in this paper. In addition, a detailed characterization of *S. typhimurium* Dam⁻ mutants shows that they share with those of *E. coli* many defects in recombination and DNA repair; however, differences are also found. The latter are intriguing and

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potentially insightful, like other examples of divergence between Escherichia and Salmonella (RILEY and SANDERSON 1990).

The last part of this study describes a genetic screening for the detection of genes regulated by Dam methylation and the finding of a locus strongly repressed by Dam methylation, located in the *S. typhimurium* virulence plasmid.

MATERIALS AND METHODS

Bacterial strains, bacteriophages and strain construction: The *S. typhimurium* strains used in this study are listed in Table 1. Transductional crosses using phage P22 HT 105/1 *int201* (SCHMIEGER 1972; G. ROBERTS, unpublished data) were used for strain construction operations involving chromosomal markers; the transducing phage will be henceforth referred as "P22 HT". Phage sensitivity was tested by cross-streaking with the clear-plaque mutant P22 H5. To obtain phage-free isolates, transductants were purified by streaking on "green" plates. Strains SV3019–SV3030 arose from matings between the *E. coli* donors CC101–CC106 (CUPPLES and MILLER 1989) and either of the *S. typhimurium* recipients SV3016 or SV3017. In these crosses, F-prime transfer was scored on E plates, selecting Pro$^+$ Tet^r or Pro$^+$ Cam^r transconjugants; growth in the absence of proline selected for F-prime transfer, while the presence of the antibiotic counterselected the *E. coli* donors. Strain UA1570, obtained from Xavier Garriga (Dep. Microbiologia, Universitat Autònoma de Barcelona, Bellaterra, Spain), carries a Tn*5* chromosomal insertion that has not been mapped; we have named this insertion *zzz-6305*, following the nomenclature of CHUMLEY *et al.* (1979) and using a *z-* allele number assigned to our laboratory by the Salmonella Genetic Stock Center (SGSC), University of Calgary, Alberta, Canada. The nature and the origin of this Tn*5* insertion are described in the following section. Using the same nomenclature system, a MudJ insertion in an unknown locus of the *S. typhimurium* virulence plasmid has been named *zzv-6306*::MudJ.

Plasmids and transposons: F'128 *pro*$^+$ *lac*$^+$ *zzf-1836*::Tn*10d*Cam is an *E. coli* episome carrying a defective Tn*10* element that confers cloramphenicol resistance (ELLIOTT and ROTH 1985). pNK972 (Amp^r) is a pBR332 derivative carrying the IS*10* transposase gene under the control of a *tac* promoter (BENDER and KLECKNER 1986). The *E. coli* episome F'128 *pro*$^+$ *lac*$^+$ *zzf-1831*::Tn*10d*Tet and the *S. typhimurium* episome F'152 *nad*$^+$ *zzf-1833*::Tn*10d*Kan were both obtained from J. R. ROTH, Department of Biology, University of Utah, Salt Lake City, Utah. Plasmid pTP166, obtained from M. G. MARINUS (Department of Pharmacology, University of Massachusetts, Worcester, Massachusetts), is a pBR322 derivative carrying the *E. coli dam* gene under the control of a *tac* promoter (MARINUS *et al.* 1984). Repression of the *tac* promoter was achieved by using the episome F' *pro*$^+$ *lacI*^{q} *L8Z*::Tn*10*Δ*4-HH104*, obtained from J. R. ROTH. Plasmid pGE108 (Kan^r) is a ColE1 derivative carrying a *cea*::*lacZ* fusion (SALLES *et al.* 1987). Plasmid pSE143 (Kan^r) is a pSC101 derivative carrying a *umuDC*::*lacZ* fusion (ELLEDGE and WALKER 1983). pIZ53 is a pUC19 derivative carrying the internal *Hin*dIII fragment of Tn*5*; this fragment includes the kanamycin-resistance gene (MALDONADO *et al.* 1992). MudI is the specialized transducing phage MudI (Amp Lac *cts*62) originally constructed by CASADABAN and COHEN (1979). MudI-8 is a transposition-defective derivative of MudI (HUGHES and ROTH 1984). MudI1734[Kan-Lac] (CASTILHO *et al.* 1984) is a transposition-deficient Mu derivative that generates operon fusions upon insertion; the element has been renamed MudJ (HUGHES and ROTH 1988). MudQ is a "locked-in" P22-Mu hybrid conferring chloramphenicol resistance (YOUDERIAN *et al.* 1988; BENSON and GOLDMAN 1992). The insertion allele *zzz-6305*::Tn*5*[lexA::*lacZ*] carries a defective Tn*5* derivative bearing a transcriptional fusion between the *S. typhimurium lexA* promoter/operator region and the *E. coli lacZ* gene (GARRIGA 1992).

Media and growth conditions: The E medium of VOGEL and BONNER (1956) was used as the standard minimal medium. NCE is E medium without citrate. Carbon sources were either 0.2% glucose or 1% lactose. The rich medium was nutrient broth (8 g/l, Difco) with added NaCl (5 g/l). MacConkey agar base was from Difco. Solid media contained Difco agar at 1.5% final concentration. Auxotrophic requirements and antibiotics were used at the final concentrations described by MALOY (1990). Green plates were prepared according to CHAN *et al.* (1972), except that methyl blue (Sigma) substituted for aniline blue. For the selection of tetracycline-sensitive derivatives of Tet^r strains, we used the medium of BOCHNER *et al.* (1980) as modified by MALOY and NUNN (1981).

Transposon substitutions: A lysate grown on strain TT10425 was used for transposon replacement at the *dam* locus. This strain carries an F prime containing Tn*10d*Kan (see the strain list). The lysate was irradiated with UV light, using a 15 W Sylvania lamp at a distance of 30 cm for 30 sec; irradiation of the phage suspensions can be expected to increase recombination in the transductants (RUPP *et al.* 1971). Transductions selecting the incoming marker (Kan^r) were carried out. Kan^r transductants were then scored for loss of the resident marker (Tet^r). This procedure allowed us to obtain allele variants tagged with different antibiotic resistance markers (*e.g.*, Tet^r and Kan^r). Replacement of a *lacZ* allele with the *lacZ477*::Tn*10d*Tet insertion was achieved using a lysate grown on strain TT16716. The lysate was UV irradiated as above; Tet^r transductants were selected on lactose indicator plates supplemented with tetracycline.

Measurement of spontaneous mutation rates using *lac* alleles: Aliquots containing 10^6 cells were added to six tubes of NCE liquid medium containing 0.2% glucose. The cultures were incubated at 37° until saturation ($\sim 10^9$ cells/ml). Cells were then spread on NCE-lactose plates (10^8 cells per plate, four plates per culture). Revertants were scored after 48-hr incubation at 37°. Viable cell counts were carried out on NB plates.

Mutagenesis with Tn*10d*Cam and isolation of insertions linked to the *dam* locus: We used the "nonhomologous transduction" procedure (ELLIOTT and ROTH 1985), with modifications described elsewhere (FLORES and CASADESÚS 1995). A lysate grown on strain SV2056 was used to transduce strain SV3002, selecting Cam^r Amp^r transductants. Transducing mixtures were made on NB plates and preincubated for 4–6 hr at 37° before replica-printing to NB supplemented with chloramphenicol and ampicillin. Cam^r Amp^r transductants were replica-printed several (more than three) times to NB-Tet plates containing EGTA 10 mM; the latter was added to prevent reinfection (thus allowing the isolation of phage-free derivatives). Pools of 1000–2000 Cam^r Tet^r colonies were made and lysed with phage P22 HT. The pools were then used to transduce strain LT2; to detect cotransduction of the Tn*10d*Cam and Tn*10d*Tet elements, Tet^r transductants were replica-printed to NB plates supplemented with chloramphenicol.

Transposition of MudJ and random formation of transcriptional *lac* fusions: A P22 HT lysate grown on strain TT10288 (HUGHES and ROTH 1988) was used to transduce TT1704, selecting kanamycin resistance. The recipient strain carried the nontransducible deletion Δ*his-9533*. Transductants were

TABLE 1

Bacterial strains

Strain	Genotype or phenotype	Reference[a]
JC608	*dam-201*::Tn*10d*Tet *rpsL1*	
JC609	*dam-202*::Tn*10d*Tet *rpsL1*	
JC610	*dam-203*::Tn*10d*Tet *rpsL1*	
SV80	Δ*his-3050*	
SV1223	*recB503*::Tn*10*	GARZON *et al.* (1996)
SV1234	*recF522*::Tn*5*	A. GARZON
SV1240	*zzz-6305*:Tn5 [*lexA*::*lacZ*]	A. GARZON
SV1244	*recA1 recB497*::MudJ	GARZON *et al.* (1995)
SV2056	Δ*his-3050*/F' 128 *lac*⁺ *pro*⁺ *zzf-1836*::Tn*10d*Cam	FLORES and CASADESÚS (1995)
SV3000	*dam-201*::Tn*10d*Tet	
SV3001	*dam-201*::Tn*10d*Kan	
SV3002	*dam-201*::Tn*10d*Tet/pNK972	
SV3003	*zzv-6306*::MudJ	
SV3004	DUP [*trp-2482* *MudI-8* *hisD9953*]	
SV3005	DUP [*trp-2482* *MudI-8* *hisD9953*] *dam-201*::Tn*10*dTet	
SV3006	Δ*dam-204*	
SV3007	*dam-201*::Tn*10d*Tet *zzz-6305*:Tn*5* [*lexA*::*lacZ*]	
SV3008	Δ*dam-204 zzz-6305*:Tn5 [*lexA*::*lacZ*]	
SV3009	LT2/pTP166	
SV3010	DUP [*trp-2482* *MudI-8* *hisD9953*] *dam*Δ*204*	
SV3011	Δ*dam-204 mutL111*::Tn*10*	
SV3012	Δ*dam-204 mutS121*::Tn*10*	
SV3013	Δ*dam-204 mutH101*::Tn5	
SV3014	Δ*dam-204*/pTP166	
SV3015	DUP [*trp-2482* *MudI-8* *hisD9953*] *dam*Δ*204 mutH101*::Tn*5*	
SV3016	*proA692*:MudQ	
SV3017	*dam-201*::Tn*10d*Tet *proA692*::MudQ	
SV3019	*proA692*:MudQ/F' *lacZ101 proAB*⁺	
SV3020	*proA692*:MudQ/F' *lacZ102 proAB*⁺	
SV3021	*proA692*:MudQ/F' *lacZ103 proAB*⁺	
SV3022	*proA692*:MudQ/F' *lacZ104 proAB*⁺	
SV3023	*proA692*:MudQ/F' *lacZ105 proAB*⁺	
SV3024	*proA692*:MudQ/F' *lacZ106 proAB*⁺	
SV3025	*dam-201*::Tn*10d*Tet *proA692*:MudQ/F' *lacZ101 proAB*⁺	
SV3026	*dam-201*::Tn*10d*Tet *proA692*:MudQ/F' *lacZ102 proAB*⁺	
SV3027	*dam-201*::Tn*10d*Tet *proA692*:MudQ/F' *lacZ103 proAB*⁺	
SV3028	*dam-201*::Tn*10d*Tet *proA692*:MudQ/F' *lacZ104 proAB*⁺	
SV3029	*dam-201*::Tn*10d*Tet *proA692*:MudQ/F' *lacZ105 proAB*⁺	
SV3030	*dam-201*::Tn*10d*Tet *proA692*:MudQ/F' *lacZ106 proAB*⁺	
SV3031	DUP [*trp-2482* *MudI-8* *hisD9953*] *mutH101*::Tn*5*	
SV3069	*zzv-6306*::MudJ *dam-201*::Tn*10d*Tet	
SV3070	*zzv-6306*::MudJ Δ*his-3050*	
SV3071	*zzv-6306*::MudJ Δ*his-3050 dam-201*::Tn*10d*Tet	
SV3072	*zzv-6306*::MudJ Δ*his-3050*	
SV3073	*zzv-6306*::MudJ Δ*his-9533 dam-201*::Tn*10d*Tet	
SV3074	*zzv-6306*::MudJ [*lacZ*::Tn*10d*Tet]	
TR5527	Δ*his-712*, cured of the virulence plasmid	J. R. ROTH
TR5667	*cysG439 rpsL1*	J. R. ROTH
TR5878	r(LT2)⁻ m(LT2)⁻ r(S)⁺ *ilv-542 met A22 trpB2* Fels2⁻ *fliA66 rpsL120 xyl-404 metE551 hspL56 hspS29*	SGSC[b]
TT1704	Δ*his-9533*	J. R. ROTH
TT2742	*aroB542*:Tn*5*	SGSC[b]
TT9286	*proAB47 leuD798 argI537 ara-9 fol-1*/F'128 *proA*⁺*B*⁺ *argF*⁺ *lacI9 Z*⁺ *Y*⁺ *A*⁺	J. R. ROTH
TT10425	*nadA56*/F'152 *nad*⁺ *zzf-1833*::Tn*10d*Kan	J. R. ROTH
TT10288	*hisD9953*::MudJ *hisA944*::MudI	HUGHES and ROTH (1988)
TT10838	*recA1*	J. R. ROTH
TT11289	*recA1 srl-203*::Tn*10d*Cam	J. R. ROTH
TT15278	*recJ504*::MudJ	MAHAN *et al.* (1992)
TT16716	*his-644 pro-621*/F' *pro*⁺ *lacZ477*::Tn*10d*Tet	SGSC[b]
UA1570	*zzz-6305*:Tn*5* [*lexA*::*lacZ*] Rif[r]	X. GARRIGA

[a] Omitted for strains first described in this study.

[b] SGSC: Salmonella Genetic Stock Center, Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada T2N 1N4.

selected on lactose indicator media (MacConkey-lactose or X-gal NB plates) supplemented with kanamycin. Rare Km^r Ap^r transductants generated by double-fragment transduction are easily identified because they are Kan^r Amp^r, while transductants carrying MudJ "hops" are Kan^r Amp^s (HUGHES and ROTH 1988). Kan^r transductants were made phage-free by replica-printing (more than two times) to plates containing 10 mM EGTA.

Transduction and cotransductional mapping: Preparation of phage P22 lysates and transductions were performed according to GARZON *et al.* (1995). The efficiency of transduction (EOT) of a given lysate is the ratio between the numbers of transductants obtained on a pair of isogenic strains. For cotransductional mapping, transductants were replica-printed to suitable plates to score transduction of unselected markers. Cotransduction frequencies are averages of more than four independent crosses, scoring at least 300 transductants from each cross. The relative order of markers was determined by three-factor crosses. Table 1 contains the original strains carrying markers cotransducible with the loci studied but not their derivatives constructed by adding one of the alleles to be mapped; such constructions were required to perform certain three-factor crosses.

UV survival assays: Overnight cultures made in NB were diluted 1/10 in the same medium. When the cultures reached an $O.D._{600}$ = 0.5, the cells were harvested and resuspended in E buffer (E medium without glucose). Five-milliliter aliquots were transferred to sterile, empty Petri dishes. Irradiation was achieved by opening the plates under a 15 W Sylvania UV lamp at a distance of 30 cm in the absence of daylight illumination. Cell suspensions were stirred during irradiation. After serial dilution in foil-covered tubes, irradiated cultures were plated on NB.

Analysis of duplication segregation: A single colony from a selective plate was used to inoculate 2 ml of nonselective NB broth, grown overnight, diluted and plated on nonselective NB agar. When colonies appeared, they were replica-printed to plates selective for cells carrying the duplication (NB supplemented with ampicillin). The percentage given refers to the fraction of colonies that lost the duplication.

Rapid mapping with MudP22 prophages: We followed the procedure of BENSON and GOLDMAN (1992), using a collection of 67 "locked-in" MudP22 prophages (BENSON and GOLDMAN 1992; FLORES and CASADESÚS 1995). This collection is not included in the strain list.

β-galactosidase assays: Levels of β-galactosidase were assayed as described by MILLER (1972), using the $CHCl_3$-sodium dodecyl sulfate permeabilization procedure.

Isolation and purification of genomic DNA: A pellet from a 5 ml culture grown in NB was resuspended in 1 ml TE-glucose (25 mM Tris-HCl, 10 mM EDTA, 50 mM glucose pH 8.0) and 0.1 ml of a mixture containing lysozyme, proteinase K and ribonuclease A (final concentrations: 5 μg/ml, 0.05 μg/ml, and 0.5 μg/ml, respectively). After 30 min of incubation at 37°, 0.1 ml SDS (20%) was added and incubation was continued for another 30 min. DNA was sheared by passing once through a syringe and extracted once with phenol, twice with phenol-chloroform and twice with chloroform. DNA was precipitated at −70° by adding 2.5 volumes of absolute ethanol and 1/10 (v:v) of 3 M sodium acetate. After centrifugation, the DNA pellet was resuspended in 0.2 ml TE buffer (Tris-HCl 10 mM, EDTA 1 mM pH 7.6).

Discrimination of the methylation status of adenine residues in genomic DNA: Genomic DNA preparations were digested with restriction enzymes *Sau*3AI, *Dpn*I and *Mbo*I (all from Boehringer Mannheim). All these enzymes recognize the sequence 5′-GATC-3′. The endonucleolytic activity of *Mbo*I is blocked by Dam methylation, while *Dpn*I only cuts methylated DNA; cutting by *Sau*3AI is irrespective of the methylation status.

Transformation of *S. typhimurium*: The transformable strain TR5878 was used as the recipient of plasmids; preparation of competent cells and transformation followed the procedures of LEDERBERG and COHEN (1974). Plasmids transformed into TR5878 were transferred to suitable recipients by transduction with P22 HT.

Extraction of the *S. typhimurium* virulence plasmid: One milliliter of an overnight culture in LB was centrifuged at 12,000 rpm for 2 min at 4°. The pellet was resuspended in 150 μl of E buffer; 300 μl of lysis solution were then added. After incubating at 65° for 1 hr, the lysate was chilled on ice and shaken for 10 min (until a white precipitate was formed). The preparation was then buffered by adding 150 μl of ice-cold 2 M Tris, shaken gently until it became transparent and centrifuged at 12,000 rpm for 20 min in the cold. The supernatant was transferred to a clean tube and mixed with one volume of nonsaturated phenol:chloroform:isoamyl alcohol (25:24:1). After two to three extraction cycles, DNA was precipitated with 3 M sodium acetate and absolute ethanol. The pellet was rinsed with 70% ethanol and resuspended in 10 μl of minimal TE. All preparations were treated with ribonuclease (0.1 mg/ml, final concentration) before storage at −20°.

DNA hybridization: DNA hybridization followed the procedures described by SAMBROOK *et al.* (1989). DNA was transferred to a nylon membrane using a vacuum blotting system (TransVac TE80, Hoeffer Scientific Instruments) and cross-linked by UV irradiation. The probes were labeled by random priming with chemiluminescent digoxigenin-dUTP (Boehringer Mannheim); hybridization bands were visualized on an X-ray film.

RESULTS

Isolation and characterization of Dam^- mutants of *S. typhimurium*: Tn*10d*Tet insertions in the *S. typhimurium dam* locus were identified following localized mutagenesis of the region known to include this gene in *E. coli*. In the latter, *dam* is located between *cysG* and *aroB* at min 74 (MARINUS 1973; BACHMANN 1990), a region that corresponds to min 75 in *S. typhimurium* (SANDERSON *et al.* 1995). In our search for Tn*10d*Tet insertions in *dam*, the *cysG* gene was used as the linked marker because mutations in the closer gene *aroB* often cause a leaky Dam^- phenotype (data not shown); a similar effect of *aroB* mutations has been described in *E. coli* (LØBNER-OLESEN *et al.* 1992). Regional mutagenesis with Tn*10d*Tet was achieved in two steps: (1) nine pools of Tn*10d*Tet insertions were generated in the wild-type strain LT2; each pool was made from 2500–3000 independent Tet^r colonies; (2) P22 HT phage grown on the pooled cells was used to transduce strain TR5667, selecting tetracycline resistance. Prototrophic ($CysG^+$) transductants were scored by replica-printing to minimal medium containing tetracycline. Selection for prototrophy eliminated potential candidates carrying *aroB* mutations polar on *dam*. The total number of Tet^r $CysG^+$ transductants obtained was 889. These were then examined for slow growth and/or abnormal colony morphology on green plates (Dam^- mutants of *E. coli* show

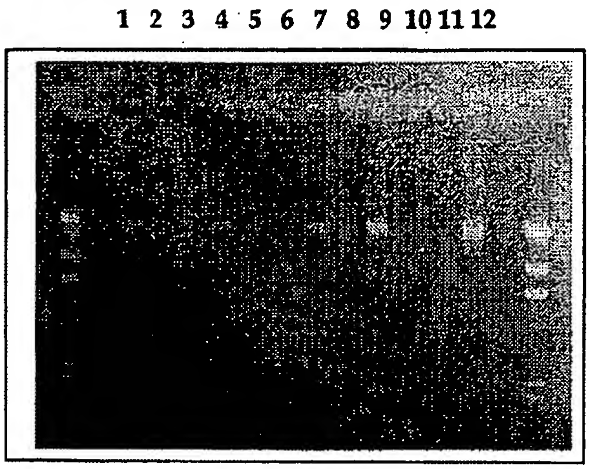

FIGURE 1.—Digestion of genomic DNAs by restriction enzymes with different responses to the methylation state of their targets (see the text for details). Lanes are as follows: 1–4, Dam$^+$ (LT2); 5–8, Dam$^-$ (SV3000); 9–12, Dam$^-$/pTP166 (SV3014). Lanes 1, 5 and 9 are controls of undigested DNA; lanes 2, 6 and 10 contain *Sau3*AI digestions; lanes 3, 7 and 11 contain *MboI* digestions; lanes 4, 8 and 12 contain *DpnI* digestions. Lanes at both gel edges contain DNA size markers (*Hin*dIII-digested lambda DNA).

abnormal cell morphology: see BARRAS and MARINUS 1989).

Twenty candidates forming abnormal colonies were physically analyzed by digestion of genomic DNA preparations with endonucleases *DpnI*, *Sau3*AI and *MboI*. All these enzymes recognize the sequence 5′-GATC-3′, but *MboI* activity is blocked by Dam methylation, while *DpnI* only cuts methylated DNA; *Sau3*AI cuts both methylated and umethylated DNA. Three independent isolates (each obtained from a different pool) proved to be Dam$^-$, as judged from their restriction patterns. These isolates were the origin of strains JC608, JC609 and JC610; their *dam* alleles were designated *dam-201*, *dam-202* and *dam-203*, respectively. The three strains looked identical in genetic and physical tests and their Southern hybridization patterns against a Tn*10* probe were identical (data not shown); thus they were probably generated by Tn*10d*Tet insertions in the same site. For further work, the allele *dam-201*::Tn*10* of strain JC608 was transduced to LT2, giving rise to strain SV3000 (see its DNA restriction pattern in Figure 1, lanes 5–8). This strain was used as the standard Dam$^-$ insertion mutant of *S. typhimurium*.

On green plates, Dam$^-$ mutants of *S. typhimurium* form flat, dull colonies that are easily distinguished from the convex, glossy colonies of the wild type (Figure 2). This distinct colony morphology is a reliable trait and can be used for strain construction.

For the generation of allele variants, a lysate grown on strain TT10425 was UV-irradiated and used to transduce SV3000. Kan^r transductants appeared at frequencies around 10^{-9} per p.f.u.; Kan^r transductants were

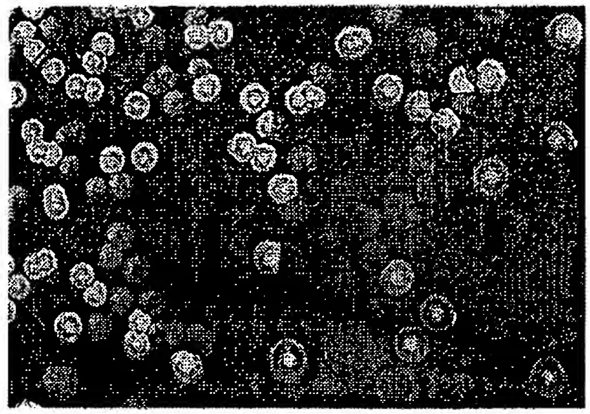

FIGURE 2.—A mixture of colonies formed by isogenic Dam$^+$ and Dam$^-$ strains of *S. typhimurium* (LT2 and SV3000, respectively) on green plates. The wild type forms glossy convex colonies, while the colonies formed by the Dam$^-$ strain are dull and flat.

scored for loss of tetracycline resistance and for the maintenance of phenotypes characteristic of Dam$^-$ mutants (flat colony shape, distinct restriction pattern of genomic DNA). One of the substitution isolates was the origin of strain SV3001.

Isolation of Tn*10d*Cam insertions linked to the *dam* locus: Pools of random Tn*10d*Cam insertions were made on strain SV3000; insertions linked to the the mutation *dam-201*::Tn*10d*Tet were detected by cotransduction, using strain LT2 as the recipient. Two independent insertions linked to the *dam* locus were obtained; the closest was *zhf-6304*, >90% linked to *dam* (see Figure 3).

Isolation and characterization of deletion mutants: Tetracycline-sensitive derivatives of strain SV3000 were isolated on Bochner-Maloy plates (BOCHNER *et al.* 1980; MALOY and NUNN 1981). Although the selection agar contains tryptone and yeast extract, *aroB* mutants are unable to form colonies on this medium unless supple-

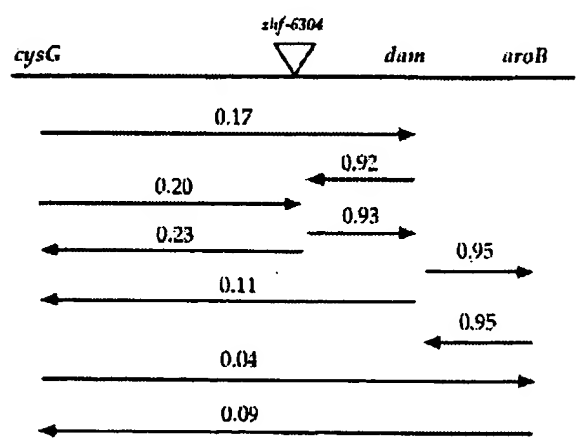

FIGURE 3.—Genetic map of the *cysG-dam-aroB* region of the *S. typhimurium* chromosome, constructed by P22-mediated transductional crosses.

mented with "aromatic mix". This selection permits the isolation of Tet^s derivatives carrying only small deletions, because their extent is constrained by *aroB* at one side and by the essential gene *trpS* at the other. One Tet^s derivative of strain SV3000 was the origin of strain SV3006; its deletion allele was named Δ*dam-204*. Genomic DNA from this strain was still able to hybridize against a *dam* probe (an *XbaI-PvuII* fragment of plasmid pTP166). However, a band shift was observed, suggesting that the *dam* gene had suffered a partial deletion (data not shown). Further evidence that Δ*dam-204* is a deletion allele was obtained during the study of a *lac* fusion repressed by Dam methylation (see below).

Complementation of *dam* mutations of *S. typhimurium* by the *E. coli dam*$^+$ gene: Plasmid pTP166 was transferred to strain TR5878 by transformation, selecting ampicillin resistance. The resulting strain, TR5878/pTP166, was used as donor to transduce pTP166 to various recipients in crosses mediated by P22 HT. When plasmid pTP166 was introduced in *S. typhimurium* Dam$^-$ mutants (*e.g.*,,SV3000 or SV3006), it complemented the *dam* mutation: all the Amp^r transductants regained both the wild-type colony shape (not shown) and a wild-type pattern of digestion by endonucleases *DpnI*, *Sau3AI* and *MboI* (see lanes 10–12 in Figure 1).

Genetic mapping of *dam* mutations: Two- and three-factor crosses were carried out by P22 HT transduction; linkage of unselected markers was scored by replica-printing. Preliminary mapping of the *dam* locus was performed using the alleles *dam-201*::Tn*10d*Tet, *dam-202*::Tn*10d*Tet and *dam-203*::Tn*10d*Tet. Further work, including strain constructions for three-factor crosses, was carried out using the alleles *dam-201*::Tn*10d*Tet and Δ*dam-204*, as well as the linked markers *aroB542*::Tn*5*, *cysG439* and *zhf-6304*::Tn*10d*Cam. The resulting genetic map is shown in Figure 3. It must be noted that linkage between *aroB* and *dam* mutant alleles must be established using the antibiotic-resistance of a Tn*10* insertion in *dam*, because the *aroB542*::Tn*5* mutation causes a leaky Dam$^-$ phenotype (data not shown).

Viability of *dam* mutations in combination with other mutations: Viability tests involved two types of transductional crosses:

(1) Insertion alleles (*e.g.*, *recD541*::Tn*10d*Cam, *recF522*::Tn*5*, *recB503*::Tn*10*, and *recJ504*::Mud*J*) were transduced to an isogenic pair of Dam$^+$ and Dam$^-$ recipients. A given combination was judged inviable whenever the frequency of transductants was >1000-fold reduced. The results of these experiments, summarized in Table 2, indicate that *dam* mutations of *S. typhimurium* are inviable if combined with *recB* mutations, but not with *recD*, *recF* or *recJ*. Note a relevant difference between *S. typhimurium* and *E. coli*: in the latter, the combination *dam recJ* is inviable (PETERSON *et al.* 1985).

(2) A lysate grown on a *recA1 srl-203*::Tn*10d*Cam donor (TT11289) was used to transduce isogenic Dam$^+$ and Dam$^-$ recipients. The *srl* and *recA* loci are 50% linked (SANDERSON and ROTH 1983). Cotransduction of *recA* and *srl* was detected by scoring UV sensitivity. The combination *recA dam* was judged inviable because 300/300 Cam^r (Srl$^-$) transductants were UV^r. Thus, as in *E. coli* (PETERSON *et al.* 1985), the combination *recA dam* is inviable in Salmonella.

In *E. coli* the inviability of *dam* mutations in combination with certain recombination functions has been attributed to the need of recombination to repair double-strand breaks derived from MutHLS-catalyzed incisions (WANG and SMITH 1986). An analogous picture can be drawn for *S. typhimurium*, because the presence of *mutH*, *mutL* or *mutS* mutations in the Dam$^-$ recipient permitted the isolation of RecB$^-$ transductants in P22-mediated crosses (Table 2). RecA$^-$ Dam$^-$ strains can be likewise constructed if the recipient contains a *mutH* allele (data not shown).

Lack and overproduction of DNA adenine methylase cause hypermutability: Rates of spontaneous mutation to rifampicin resistance were compared by plating early stationary cultures on NB plates supplemented with rifampicin. The frequency of Rif^r mutants increased from 10^{-8} in the wild type to $9–15 \times 10^{-8}$ in Dam$^-$ strains. However, the highest mutation rates (>400-fold higher than the wild type) were observed when the *E. coli dam* gene was introduced into *S. typhimurium* on a multicopy plasmid. Thus lack of Dam methylase causes a milder hypermutability than Dam overproduction, suggesting that faster DNA remethylation perturbs mismatch repair more severely than the absence of methylation. Similar results have been reported for *E. coli* (MARINUS *et al.* 1984).

The mutation pattern of Dam$^-$ strains was examined using the *lacZ* allele collection constructed by CUPPLES and MILLER (1989). Six F' *lac pro*$^+$ episomes, each containing a known base substitution in the *lacZ* gene, were introduced in isogenic Dam$^+$ and Dam$^-$ strains of *S. typhimurium*. The six *lacZ* alleles cover all six possible base substitutions (CUPPLES and MILLER 1989); the genotypes of the strains used (SV3019–SV3030) are given in the strain list. The presence of a *dam* mutation in the background increased around eightfold the reversion rates of two alleles (*lacZ102* and *lacZ106*) but had little or no effect on the other members of the *lacZ* allele collection. These results indicate that the increase of Lac$^+$ reversion observed in a Dam$^-$ host is clearly biased toward transition mutations, as in *E. coli* (GLICKMAN 1979).

Effect of *dam* mutations on SOS induction: SOS activity was tested in *lexA*$^+$/*lexA*::*lacZ* merodiploids (strains SV1240, SV3007 and SV3008), constructed by using a Tn*5*-borne *lexA*::*lac* fusion (GARRIGA 1992). Measurements of β-galactosidase activity indicated that *dam* mutations of Salmonella, like their *E. coli* counterparts (PE-

TABLE 2

Viability of *S. typhimurium dam* mutations in combination with other mutations

Transduced allele	Recipient	Genotype of the recipient	EOT[a]
recB497::Mud*J*	LT2	*dam*$^+$	1
	SV3000	*dam-201*::Tn*10d*Tet	$<10^{-3}$
	SV3006	Δ*dam-204*	$<10^{-3}$
	SV3011	Δ*dam-204 mutL111*::Tn*10*	0.33
	SV3012	Δ*dam-204 mutS121*::Tn*10*	0.45
recB503::Tn*10*	SV3013	Δ*dam-204 mutH101*::Tn*5*	1.2
recD541::Tn*10d*Cam	LT2	*dam*$^+$	1
	SV3000	*dam-201*::Tn*10d*Tet	1.51
recF522::Tn*5*	LT2	*dam*$^+$	1
	SV3000	*dam-201*::Tn*10d*Tet	0.21
recJ504::Mud*Cam*	LT2	*dam*$^+$	1
	SV3000	*dam-201*::Tn*10d*Tet	0.49

[a] EOT (efficiency of transduction) is the ratio between the numbers of transductants obtained on a pair of isogenic strains.

TERSON *et al.* 1985), undergo moderate (two- to threefold) derepression of *lexA* transcription. Derepression was not only observed for *lexA;* plate tests with plasmid-borne *umuDC::lac* and *cea::lac* fusions (ELLEDGE and WALKER 1983; SALLES *et al.* 1987) also showed increased expression in a Dam$^-$ background (data not shown). Thus *S. typhimurium dam* mutations cause moderate SOS induction, like those of *E. coli* (PETERSON *et al.* 1985).

UV sensitivity assays: Dam$^-$ mutants of *E. coli* are slightly UV-sensitive at low UV doses (GLICKMAN *et al.* 1978), but their UV sensitivity increases with respect to wild type at higher UV doses (MARINUS and MORRIS 1974). We examined the UV sensitivity of *S. typhimurium* Dam$^-$ isolates and observed a difference with their *E. coli* counterparts: Dam$^-$ mutants of Salmonella are UV-resistant at both low and high UV doses (Figure 4). Given the differences in UV mutability between *E. coli* and Salmonella (reviewed by EISENSTADT 1987) and the presence of *umuDC*-like genes in the *S. typhimurium* virulence (sometimes called "cryptic") plasmid (NOHMI *et al.* 1991), we examined the possibility that the UV resistance of *S. typhimurium* Dam$^-$ mutants might involve plasmid-borne function(s). For this purpose, we constructed a Dam$^-$ derivative of a strain cured of the virulence plasmid and compared its UV sensitivity with that of the parental strain, TR5527. Both UV sensitivity curves were similar and roughly identical to those of Dam$^+$ and Dam$^-$ LT2 derivatives shown in Figure 4. Thus Dam$^-$ strains of *S. typhimurium* may be intrinsically resistant to UV radiation. Or, if alternative functions exist, they must lie in the chromosome and not in the virulence plasmid.

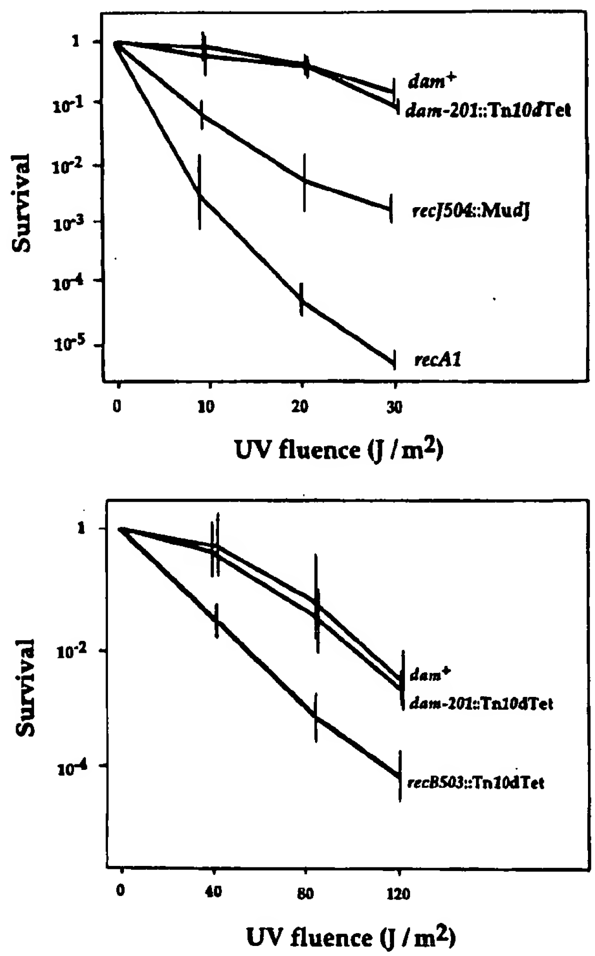

FIGURE 4.—UV sensitivity curves of Dam$^+$ and Dam$^-$ strains of *S. typhimurium* (LT2 and SV3000, respectively). To illustrate the UV sensitivity levels, three isogenic strains carrying recombination mutations are also included (TT15278, TT10838 and SV1223).

Effect of *dam* mutations on duplication segregation: Duplication segregation was examined using a test for homogenote formation formally similar to that of MARINUS and KONRAD (1976); the only difference is

TABLE 3

Effect of *dam* mutations on the segregation of chromosomal duplications

Strain	Genotype	% of segregants[a]
SV3004	DUP [*trp-2482* *Mudl-8* *hisD9953*]	67
SV3005	DUP [*trp-2482* *Mudl-8* *hisD9953*] *dam-201*::Tn*10*dTet	97
SV3010	DUP [*trp-2482* *Mudl-8* *hisD9953*] Δ*dam-204*	99
SV3031	DUP [*trp-2482* *Mudl-8* *hisD9953*] *mutH101*::Tn*5*	33
SV3015	DUP [*trp-2482* *Mudl-8* *hisD9953*] Δ*dam-204* *mutH101*::Tn*5*	40

[a] Percentage of Amp^s colonies; average of three experiments.

that we used chromosome merodiploids instead of F-prime heterogenotes. Isogenic Dam$^+$ and Dam$^-$ pairs of strains carrying Mu*d*-induced duplications with known endpoints were grown nonselectively in NB until saturation. Absence of antibiotic selection permits segregation of the Mu*d*-held duplication (HUGHES and ROTH 1985). Colonies were isolated on NB plates and haploid segregants lacking the Mu*d*-encoded antibiotic resistance (Amp^s) were detected by replica-printing (FLORES and CASADESUS 1995).

Table 3 shows that duplication segregation increases in a Dam$^-$ background; the increase can be suppressed by a *mutH* mutation. These results suggest that *dam* mutations enhance duplication segregation via mismatch repair, probably because the MutH endonuclease introduces nicks or double-stranded breaks in unmethylated DNA. Because the activated form of MutH can cleave both DNA strands at an unmethylated 5'-GATC-3' site (AU *et al.* 1992), rare mismatches generated during DNA replication can trigger MutHLS activity. The free DNA ends generated can then be used to initiate homologous recombination, as proposed for *E. coli* (MARINUS and KONRAD 1976).

A genetic screening for the detection of *lac* transcriptional fusions regulated by DNA adenine methylation: Kan^r transductants generated by Mu*dJ* insertion were classified according to their Lac phenotype; 100–500 insertions of the same class (Lac$^+$ or Lac$^-$) were then pooled and lysed with P22 HT. The lysates were used to transduce a Dam$^-$ recipient (SV3000), selecting Kan^r transductants on indicator plates. This procedure can be expected to permit the detection of fusions in genes whose transcription is regulated by DNA adenine methylation:

(1) Lac$^-$ isolates that turn Lac$^+$ in a Dam$^-$ background carry fusions putatively repressed by Dam methylation.

(2) Lac$^+$ isolates that turn Lac$^-$ in a Dam$^-$ background carry fusions putatively activated by Dam methylation.

To be classified as isolates carrying a fusion regulated by Dam methylation, candidates are required to pass three additional tests:

(1) A "backcross" in which the original Dam$^+$ isolate is made Dam$^-$ by transduction of the insertion *dam-201*::Tn*10d*Tet. This test intends to confirm that the change in color is caused by the *dam* mutation (and not by another mutation carried by the Dam$^-$ recipient).

(2) Reconstruction crosses, in which the fusion is transduced to isogenic Dam$^+$ and Dam$^-$ derivatives (LT2 and SV3006, respectively). Reconstruction in recipients different from the pair of original strains intends to confirm that the fusion is regulated by Dam methylation irrespective of the background of the recipient.

(3) Complementation with a cloned *dam*$^+$ allele (carried on plasmid pTP166). This test can confirm that the fusion is regulated by Dam methylation (and not by a gene located downstream of *dam*). This test is necessary whenever the *dam* alleles used are potentially polar (insertions or out-of-frame deletions).

Genetic characterization of a fusion repressed by Dam methylation: Among the candidates obtained with the screening described in the former section, a *lac* fusion repressed by Dam methylation was chosen for further study. Dam$^+$ isolates carrying this fusion form white colonies on MacConkey lactose, while Dam$^-$ isolates form red colonies. In a Dam$^-$ host, the presence of plasmid pTP166 causes repression of the fusion to levels similar to the wild type (data not shown). Histograms of β-galactosidase activities are shown in Figure 5. The activity of the fusion is 10-fold higher in a Dam$^-$

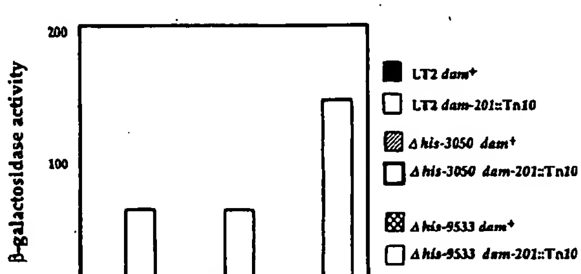

FIGURE 5.—β-galactosidase activities of isogenic pairs of Dam$^+$ and Dam$^-$ strains carrying the *zzv-6306* Mu*dJ* fusion in different backgrounds: *his*$^+$ *dam*$^+$ (SV3003), *his*$^+$ *dam*$^-$ (SV3069), Δ*his-3050* *dam*$^+$ (SV3070), Δ*his-3050* *dam*$^-$ (SV3071), Δ*his-9533* *dam*$^+$ (SV3072) and Δ*his-9533* *dam*$^-$ (SV3073).

background, a derepression level similar to that of the IS*10* transposase gene (ROBERTS *et al.* 1985) and higher than those of other Dam-repressed genes such as *glnS*, *trpR* and *sulA* (MARINUS 1985, 1987a,b; PETERSON *et al.* 1985). The fusion is not inducible by DNA damage, as indicated by treatments with mitomycin C and nalidixic acid (data not shown).

Isolates carrying the fusion in a Δ*dam-204* background are stable and do not segregate Lac^- colonies or sectors. In contrast, isolates carrying the fusion in a *dam-201*::Tn*10d*Tet background yield colonies containing Lac^- sectors at low frequency (<1%). Sectored colonies are not found if the plates contain tetracycline. These results suggest that the *dam-201*::Tn*10d*Tet insertion can undergo a low but detectable rate of excision; in fact, transposase-independent Tn*10* excision has been shown to be enhanced in Dam^- mutants of *S. typhimurium* (HAFNER and MACPHEE 1991). Most Lac^- sectors seem to be formed by Dam^+ revertants, as judged from the colony shape of purified Lac^- isolates and from their ability to inherit a *recB* mutation (data not shown). These observations provide further evidence that the allele Δ*dam-204* cannot revert (and thus is a deletion allele).

The fact that the fusion had been isolated in strain TT1704, the usual recipient for transductional delivery of Mu*dJ* (HUGHES and ROTH 1988), prompted a second, unexpected observation: in the TT1704 background, the ratio of β-galactosidase activities found in Dam^- and Dam^+ hosts is 26. This result suggests that TT1704 carries a second mutation that activates the fusion in the absence of Dam methylation. This (hypothetical) mutation has no effect in a Dam^+ background (Figure 5). The deletion carried by TT1704 (Δ*his-9533*) is nontransducible by P22; thus it must exceed the packaging capacity of the P22 capsid, 43 kb (CASJENS and HAYDEN 1988). Smaller deletions such as Δ*his-3050* do not increase the expression of the fusion (Figure 5). Thus we hypothesize that a locus located near the histidine operon may corepress the expression of the fusion.

For mapping with the "locked-in" Mud-P22 procedure (BENSON and GOLDMAN 1992), a Tn*10d*Tet element was introduced by homologous recombination in the *lacZ* gene of the Mu*dJ*-generated fusion. For this cross, a P22 lysate grown on strain TT16716 was UV-irradiated and used to transduce SV3003, selecting tetracycline resistance on X-gal plates. Four Tet^r Lac^- transductants were obtained. All carried a Tn*10* insertion 100% linked to the resident Mu*dJ* element. One of these isolates was the origin of strain SV3074.

Attempts to map the Tet^r insertion (and thus the Mu*dJ* fusion) with the locked-in Mud-P22 procedure (BENSON and GOLDMAN 1992) did not provide patches of Tet^r transductants, suggesting that the locus might not map on the chromosome. Evidence that the fusion mapped on the *S. typhimurium* virulence plasmid was

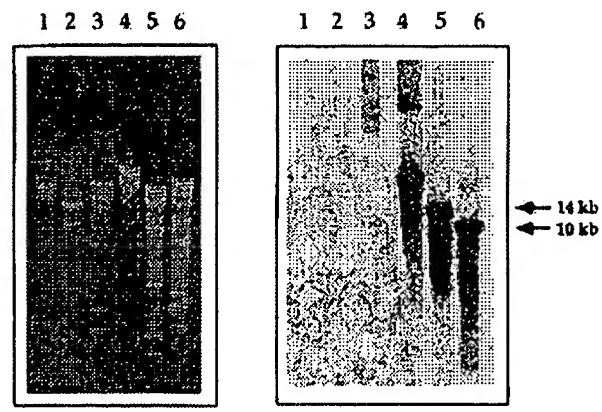

FIGURE 6.—(A) Agarose gel electrophoresis of virulence plasmid DNA preparations from strains LT2 and SV3003. Lanes are as follows: (1) virulence plasmid from LT2, undigested; (2) virulence plasmid from LT2, *Eco*RI-digested; (3) virulence plasmid from LT2, *Hin*dIII-digested; (4) virulence plasmid from SV3003, undigested; (5) virulence plasmid from SV3003, *Eco*RI-digested; (6) virulence plasmid from SV3003, *Hin*dIII-digested. (B) Southern hybridization of the DNA fragments shown in A, using the *Hin*dIII fragment of plasmid pIZ53 as a probe; this fragment contains the Tn*5* kanamycin resistance gene (which is the same Kan^r determinant carried by the Mu*dJ* element).

obtained in transductional crosses: when the original Mu*dJ* element or its Tn*10*-containing derivative were transduced to the wild-type LT2, transductants were obtained at frequencies near 10^{-5} per p.f.u. In contrast, no transductants were obtained when the recipient was a strain cured of the virulence plasmid (TR5527).

Physical mapping confirmed that the Mu*dJ*-generated fusion was located on the *S. typhimurium* virulence plasmid. DNA preparations of the latter were digested with *Eco*RI or *Hin*dIII and hybridized against the kanamycin gene of the Mu*dJ* element. The results confirmed that the fusion is plasmid-borne: the Mu*dJ* probe hybridized with a ~14-kb *Eco*RI fragment (Figure 6B, lane 5), with a ~10-kb *Hin*dIII fragment (Figure 6B, lane 6), and with undigested virulence plasmid DNA (Figure 6B, lane 4). The fusion has been named *zzv-6306*::Mu*dJ*.

DISCUSSION

A *S. typhimurium* point mutant lacking DNA adenine methylase was described a decade ago (RICHTIE *et al.* 1986) and specific aspects of Dam methylation in Salmonella have been since then investigated (RICHTIE *et al.* 1988; HAFNER and MACPHEE 1991). Although the pioneering relevance of these studies must be emphasized, their use of point mutants can be viewed as a potential source of problems. Because *dam* mutations are deleterious and highly pleiotropic, Dam^- mutants are prone either to revert or to accumulate partial suppressors (MACGRAW and MARINUS 1980); thus periodical reconstruction of Dam^- mutants is highly advisable.

This can be readily achieved by the use of insertion alleles such as the Tn*10d*Tet insertions described in this work. In turn, certain operations of strain construction are made easier by the use of deletion alleles that lack the Tn*10*-encoded tetracycline resistance. Whenever necessary, Dam$^-$ deletion strains can be reconstructed using nearby insertions such as *zhf-6304*::Tn*10d*Cam. For strain construction, one useful phenotype of Dam$^-$ mutants of *S. typhimurium* is their abnormal colony morphology on green plates. Like other phenotypes of Dam$^-$ strains, the unusual aspect of their colonies tends to disappear upon repeated subculture, but it is a reliable trait to score for Dam$^-$ transductants. Confirmation of the *dam* genotype may require either a genomic DNA digestion test or a compatibility assay with *recA* or *recB* mutations (see below).

Tn*10d*Tet insertions in the *dam* locus of *S. typhimurium* map at 75 min, as previously reported (RICHTIE *et al.* 1986). Cotransductional mapping shows that the *dam* locus is 10% linked to *cysG* and 95% linked to *aroB;* these data suggest a position identical to that of the *E. coli dam* gene (MARINUS 1973; LYNGSTADAAS *et al.* 1995). The structure of the *dam* gene itself is likely to be conserved, because many (if not all) phenotypes of Salmonella Dam$^-$ mutants are efficiently complemented by a plasmid containing the *E. coli* wild-type *dam* gene. A corollary is that the phenotypes that can be complemented are unequivocally caused by lack of DNA adenine methylase (and not by a polar effect of the *dam* mutation on downstream genes).

Deletion alleles were obtained by selecting Tet^s derivatives of Tn*10d*Tet insertions in *dam*. Deletions that remove genetic material from *dam* toward *cysG* are necessarily small because of the presence of the essential gene *trpS* (LYNGSTADAAS *et al.* 1995; SANDERSON *et al.* 1995). At the other side of *dam*, the extent of deletions can be constrained by taking advantage of the presence of the *aroB* gene: if Tet^s derivatives are obtained on plates lacking an aromatic mix supplement, only AroB$^+$ Tet^s derivatives are obtained. The advantage of these constraints is the yield of deletions (such as Δ*dam-204* and others) that remove little genetic material around *dam* and thus are safe to identify the consequences of loss of the Dam function (and not the combined effects of multigenic losses).

Attempts to construct double mutants containing a *dam* mutation and another mutation affecting recombination and/or repair has indicated that the combinations *recA dam* and *recB dam* are inviable, while the combinations *recD dam, recF dam* and *recJ dam* are viable. These data indicate that viability of Dam$^-$ strains of *S. typhimurium* requires certain recombination functions (RecA and RecBC) while others are dispensable (RecD, RecJ and RecF). By analogy with *E. coli*, one may interpret this to mean that Dam$^-$ strains suffer a higher incidence of MutH-catalyzed double strand breaks (WANG and SMITH 1986). This view is supported by the observation that Dam$^-$ RecA$^-$ and Dam$^-$ RecB$^-$ mutants of *S. typhimurium* are viable in the presence of mutations that eliminate mismatch repair such as *mutH, mutL* or *mutS*. Thus the RecA and RecBCD functions seem to be involved in the repair of the double-strand breaks typical of Dam$^-$ strains of Salmonella. For practical purposes, the incompatibility between *recB* and *dam* mutations permits a rapid identification test: any putative Dam$^-$ construct can be identified as such if it fails to inherit a *recB* insertion allele in a P22-mediated transductional cross (well-characterized insertions in *recA* are not available in *S. typhimurium*).

An important difference between Dam$^-$ mutants of *E. coli* and Salmonella concerns RecJ function: Dam$^-$ RecJ$^-$ mutants are viable in *S. typhimurium* but not in *E. coli* (PETERSON *et al.* 1985). A recent study has suggested that repair of double-strand breaks in methylation-deficient strains of *E. coli* requires a RecBC-dependent pathway that includes RecJ (PETERSON and MOUNT 1993). In contrast, our data indicate that RecJ is not necessary for viability of Dam$^-$ strains of Salmonella, at least in an SbcB$^+$ background. Although the difference is surprising, one should keep in mind that the RecJ product may play slightly different roles in *E. coli* and Salmonella (MAHAN *et al.* 1992).

Another unexpected difference between Dam$^-$ mutants of *E. coli* and Salmonella is UV sensitivity: unlike *E. coli, S. typhimurium* Dam$^-$ mutants are not UV-sensitive (not even at high UV doses: see MARINUS and MORRIS 1973; GLICKMAN *et al.* 1978). This observation suggests that methyl-directed DNA repair does not play a significant role in the repair of UV damage in Salmonella. This observation is supported by the observation that *mut* alleles do not cause UV sensitivity (SHANABRUCH *et al.* 1981). However, we were surprised by the difference between *E. coli* and Salmonella and considered the possibility that Salmonella might have alternative repair functions. An obvious genetic entity that might harbor those hypothetical functions is the *samAB*-containing virulence plasmid (NOHMI *et al.* 1991). However, Dam$^-$ mutants proved to be still UV^r in the absence of the virulence plasmid. Thus, if alternative functions exist, they must map elsewhere.

Aside from the differences discussed above, Dam$^-$ mutants of *S. typhimurium* share with those of *E. coli* many relevant phenotypes: (1) increased incidence of spontaneous mutations, biased toward transitions (GLICKMAN 1979); (2) severe hypermutability (MARINUS *et al.* 1984); (3) moderate SOS induction (PETERSON *et al.* 1985) and (4) enhanced duplication segregation, which can be suppressed by mutations that eliminate mismatch repair (MARINUS and KONRAD 1976). The balance of analogies and differences indicates that Dam methylation performs similar roles in *E. coli* and Salmonella. Moreover, the ability of the *E. coli dam* gene to

complement *S. typhimurium dam* mutations suggests that Dam methyltransferase may be a function highly conserved among both taxa. On the other hand, the differences found are less surprising if one considers that the genera Escherichia and Salmonella diverge in other aspects of recombination and repair (EISENSTADT 1987).

Genes regulated by Dam methylation have been traditionally discovered by reverse genetics, after the presence of 5′-GATC-3′ sites in or near the promoter (MARINUS 1987; NOYER-WEIDNER and TRAUTNER 1993). In contrast, classical genetic strategies like the *lac* fusion screening described above can identify novel, uncharacterized loci regulated by Dam methylation. Our screening has two caveats: (1) it relies on the use of unmethylated DNA that, in general, is a nonphysiological condition; thus it might give rise to artefacts whenever hemimethylation and unmethylation are not equivalent signals; (2) small differences in transcriptional activity may not be easily seen on plate tests. Despite these potential limitations, the screening has already proved useful to detect a locus repressed by Dam methylation, located in the *S. typhimurium* virulence plasmid.

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